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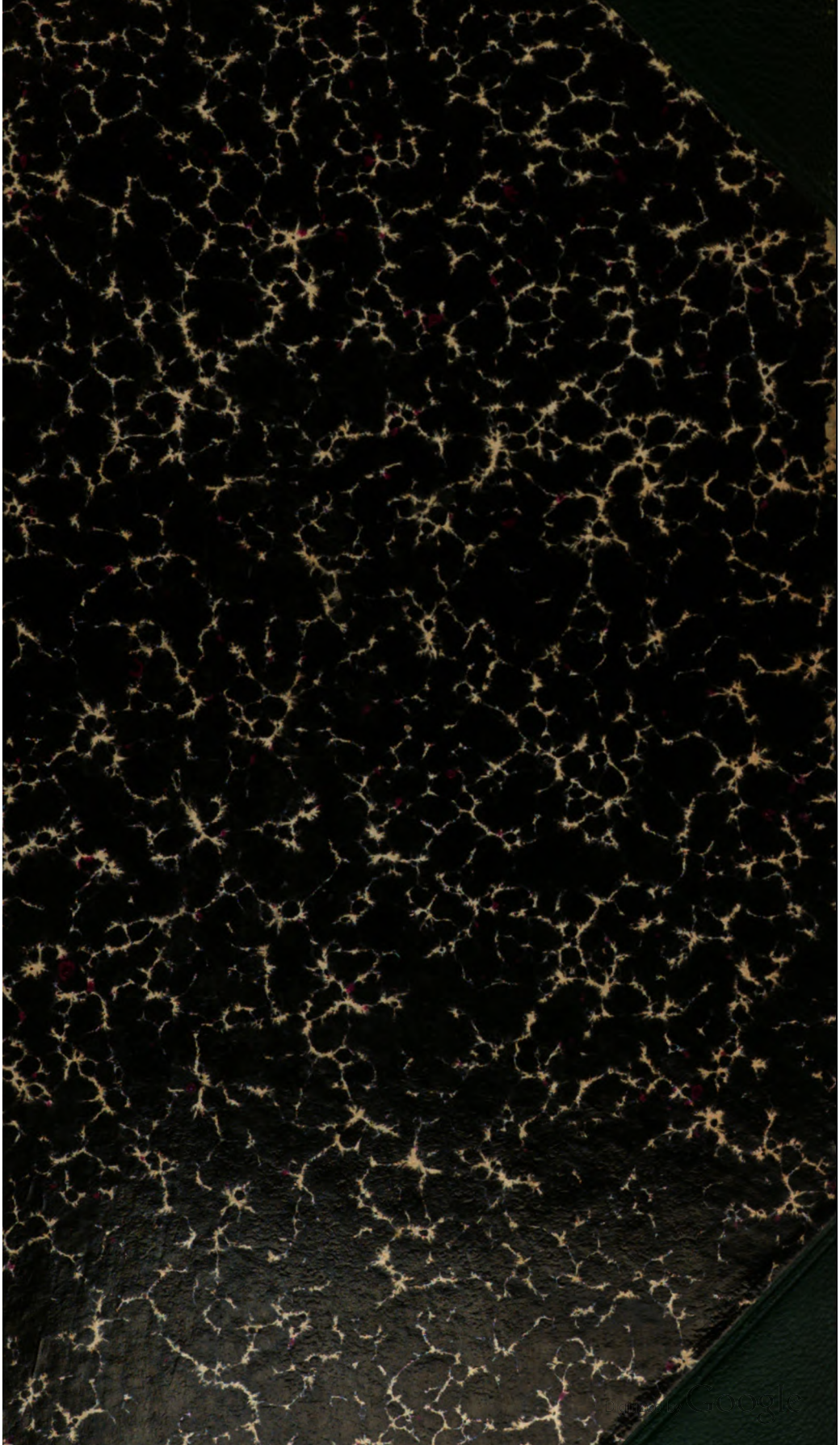

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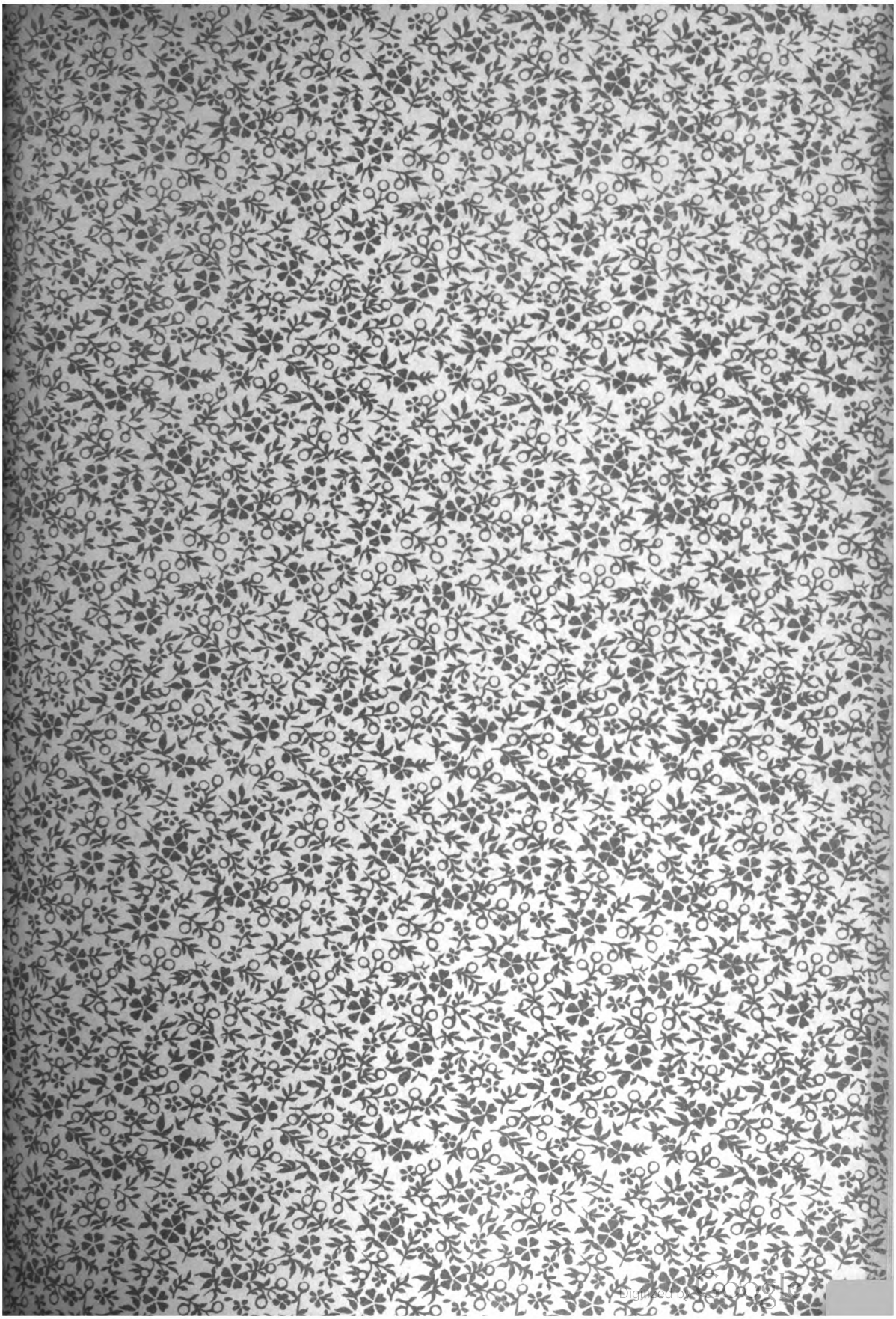

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EDITORS

JACQUES LOEB W. J. V. OSTERHOUT

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PHOTOCHEMISTRY OF VISUAL PURPLE.

I. The Kinetics of the Decomposition of Visual Purple by Light.

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(Received for publication, July 24, 1920.)

I.

An analysis of the progress of retinal dark adaptation in man has shown that the process follows the course of a bimolecular reaction (Hecht, 1919–20). The interpretation to be placed on these findings is that dark adaptation depends on the disappearance in the retina of *two* products of decomposition resulting from the photolysis of a sensitive material. This rests on the assumption that the sensitivity of the peripheral retina measured in terms of the threshold stimulus is directly proportional to the quantity of residual decomposition products then present in the retina.

A photosensitive substance undoubtedly exists in the irritable elements, and its decomposition by light must form the first step in the receptor process. It may be supposed that this sensitive substance breaks down into two products of decomposition; and that in the dark these two products reunite to form the sensitive material again. This reversible reaction may be expressed as

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A$$

in which S is the sensitive substance, P is the principal precursor decomposition product, and A its accessory. Such a scheme has been shown to fit the facts of retinal adaptation, and to offer a starting point for quantitative investigations into the mechanism of visual reception (Hecht, 1919–20).

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In terms of such an hypothesis of photoreception it would be a distinct step forward to be able to identify the three components of the reaction, and to discover their exact chemical interrelations. At present no definite statement is possible in this connection. However, dark adaptation is decidedly a phenomenon of dim vision. According to the Duplicity Theory, dim vision is associated with the rods of the retina. The rods contain a photosensitive substance known as visual purple. It is therefore *possible* that dim vision and, more particularly, dark adaptation are in some way conditioned by the properties of visual purple.

The association of visual purple with dim vision rests on more than the mere possibility just stated. A comparison of the threshold of visual sensibility at different wave-lengths with the spectral absorption of visual purple and its photochemical sensitivity has shown that these three sets of data resemble each other to a striking degree (Trendelenburg, 1911). It is similarly with the relation between dark adaptation and visual purple. A retina exposed to light is bleached. Placed in the dark, the intact eye or the retina alone with its pigment layer will regenerate the purple color. Under similar conditions the living eye becomes dark-adapted. The presumption is therefore that these phenomena are in some way correlated.

Our judgment in this matter must, however, be withheld, because no quantitative data are available with regard to the photochemistry of visual purple. Ever since Boll's (1876) discovery of this substance, and Kühne's classic qualitative investigations with it (Ewald and Kühne, 1878), there have been but a few scattered observations made with visual purple. A notable exception is the work of Trendelenburg (1904). It, however, has been barely a beginning.

It has therefore been necessary to investigate the visual purple mechanism from the modern point of view of photochemistry, and to attempt a complete analysis of the reactions concerned. The present paper is the first of a series in which the results of this investigation are to be presented. As a product of these researches, I hope to be able first, to decide whether visual purple is really intimately concerned with dark adaptation and the reception of dim lights, and, second, if it is so concerned, to determine precisely the way in which this is brought about.

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II.

1. Thanks to the work of Ewald and Kühne, the preparation of visual purple is a simple matter. For my purposes the following routine has been adopted. Thirty frogs are placed over night in complete darkness. The next morning the heads are removed and placed in salt solution. The eyes are then enucleated, cut in two, and the retinas removed as free from the pigment layer as possible. This is much facilitated if the frogs have been kept over night in a warm room. The retinas with some salt solution are now placed in a centrifuge tube, and centrifuged at a good speed for 30 minutes. The supernatant liquid is removed, and a 3 to 4 per cent solution of bile salts is added to the retinas. About 5 cc. are usually added, but this may be varied up to 20 cc. depending on the concentration desired. The retinas are thoroughly stirred up, and are allowed to remain in suspension for half an hour or more. The mixture is then again centrifuged. This brings the disrupted retinas and stray pigment granules to the bottom of the tube, whereas the supernatant liquid is a clear purplish pink solution of visual purple. In this form it may be kept on ice for as long as a week or more without deterioration. It can be used directly or after dilution with water or bile salts solution.

All the manipulations are carried out in the dark, with the aid of the light from a 10 watt ruby lamp. This is hardly a source of error, because the solutions are quite insensitive to such red light. However, even to this light, they are exposed as little as possible. In considering sources of error it may be well to mention the purity of the bile salts. The commercial preparations are hopeless. The bile salts which I used were prepared in our own laboratory, and are a white powder, which when dissolved in water gives an absolutely colorless solution. Only such bile salts give reliable results.

A solution of visual purple prepared in this manner is extremely sensitive to light. If exposed to sunlight it loses its color in about a second. The solution, however, is not bleached to a completely colorless condition. The bleached solution is slightly but definitely yellow, and remains so no matter how long the exposure is maintained. In this I can confirm Trendelenburg (1904) as opposed to Ewald and Kühne's original statement (1878, p. 181) that the solu-

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tion first turns yellow, and after some time turns completely colorless (*wasserhellig*). The discrepancy between the two observations I have not yet been able to explain.

The velocity of visual purple bleaching varies with the intensity; the rate may thus be controlled to allow a quantitative following of the process. With intensities near 100 meter candles the solutions may take half an hour to bleach. The purplish pink solution slowly loses its color, at the same time taking on a yellowish tinge, which increases and remains. The solutions obtained by the present technique do not show a reversible reaction. The bleached solutions retain their yellowish color in the dark for as long as they can be kept from bacterial decomposition. The conditions under which reversal takes place are different in several respects, and will be considered at length in future contributions.

2. The most obvious means of following the process is a colorimetric one. Unfortunately only a small quantity of material is available, and in dilute solution at that. A method has therefore been devised by means of which small amounts of solutions are manipulated accurately, and are made to produce the same optical effects as large quantities. The experiments are performed in very small test-tubes, made by sealing one end of short lengths of narrow bore glass tubing. The tubes are about 50 mm. long, and have an inside diameter of 2 mm., and an outside diameter of 2.5 mm. Each test-tube holds a little over 0.1 cc. This quantity of solution produces a depth of nearly 35 mm. Viewed end-on, the effect is produced of a solution of much greater concentration.

The crucial point is, of course, to have equal depths of liquid in all tubes of any given set of experiments. This is accomplished very simply. The test-tube is placed into a wider tube which fits it snugly. One end is sealed, and near the open end is a circular scratch. The inner tube rests on the bottom of the measuring tube. The solution is now inserted into the inner tube by means of a pipette having a long capillary tip, and the depth of the solution is so adjusted that the bottom of the meniscus is on a line with the circular scratch. This can easily be done within 0.2 mm., and, including slight variations in the thickness of the sealed end of the test-tube, would make the error in this part of the manipulation of the order of 1 per cent.

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3. In order to follow quantitatively the progress of the bleaching reaction, it is necessary to have a series of colorimetric standards representing different concentrations of visual purple and its decomposition products. Eleven such concentrations are made up, representing on the one extreme 100 per cent of the unbleached substance plus 0 per cent of the bleached; and on the other extreme, 0 per cent of the unbleached plus 100 per cent of the bleached, the concentrations changing by 10 per cent steps. Actually these are made up in dry test-tubes by taking a total of 20 drops of solution: the first tube having 20 of the unbleached; the next tube 18 of the unbleached plus 2 of the bleached; the next 16 of the unbleached plus 4 of the bleached, etc. The same dropping pipette is used each time to insure uniformity.

The experimental unbleached solution is pipetted into the small exposure tubes. Into similar tubes and under the same conditions are pipetted the standard concentrations just described. The standards are jacketed with thick, black rubber tubing, except for a few millimeters at each end. In this way a much clearer end-on view is obtained. The exposure tube containing the experimental solution of visual purple is similarly jacketed when colorimetric comparisons are to be made. The matching is done against an artificial daylight lamp of 0.04 candle power, having a uniform circular surface 30 mm. in diameter. Such a light is sufficiently bright for work in the dark, especially after one has become dark-adapted. The matching light is turned on and off by a spring contact controlled by the observer's foot, so that only a momentary exposure is made.

When not in actual use the standards are kept under cover even in the dark room. As a rule, fresh standards are made daily. If they have been used but little, they may be kept on ice until the next day, provided they are placed in a moist atmosphere so as to prevent evaporation. They suffer comparatively little deterioration from their use, mainly because the exposures to the daylight lamp are so brief. After continuous use for several days, they are definitely off; hence the practice of making fresh standards daily.

4. The experimental tube is exposed to light of known intensity. The apparatus used for this purpose is shown full size in Fig. 1. The glass rod to which the exposure tube is attached is rotated by hand at a rate depending on the intensity of the light. For the experi-

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ments reported in this paper this was roughly ten revolutions per minute. By this means an even exposure is obtained for this thin column of solution. This obviates the necessity for stirring the solu-

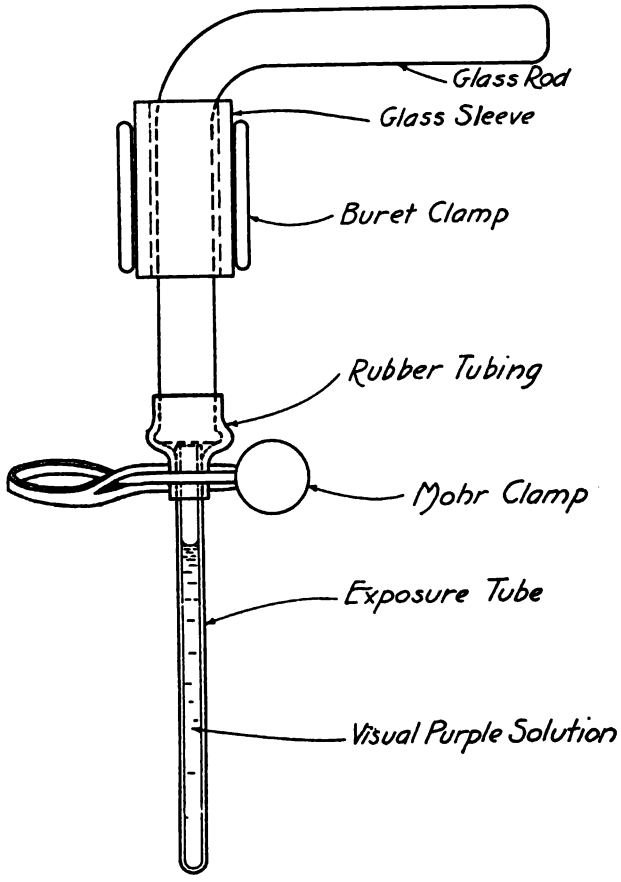

FIG. 1. Full size diagram of apparatus used for the exposure of visual purple solutions to light. The exposure tube being attached to the glass rod, it is possible to rotate the tube on its own axis by rotating the glass rod.

tion, and removes all errors associated with unequal illumination, diffusion, etc. (*cf.* Sheppard, 1914, p. 239). In this respect the small quantities of material and the thin test-tubes are of distinct advantage. After the proper length of exposure the light is turned off, the tube

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removed, jacketed with the rubber tubing, and compared with the standards.

Readings are usually made to the nearest 5 per cent concentration. The reading is thus, say, 70 or 75 per cent. Occasionally, however, it is so clear that the color is nearly but not quite one of the 10 per cent tubes, that the reading is given between the 5 and the 10 per cent

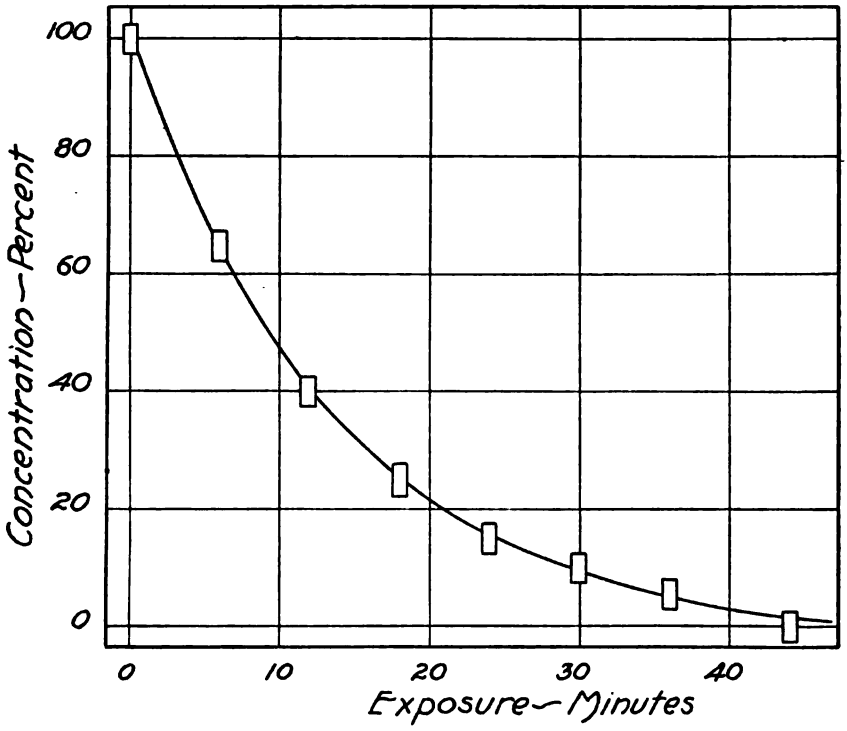

FIG. 2. The results of Experiment 80. Light intensity is 53 meter candles. The monomolecular nature of the bleaching process is evident from the coincidence of the experimental points (rectangles) with the curve calculated on the assumption that $k = 0.034$.

steps; for example, 48 or 52. This happens comparatively infrequently.

After a colorimetric determination the jacket of the tube is removed, the tube replaced, and the exposure renewed. After a sufficient exposure, the tube is again removed, and its concentration again determined. The process is repeated until the bleaching is complete.

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III.

1. Altogether I have followed the progress of the bleaching of visual purple in 88 separate experiments. These have been performed under a variety of conditions to which reference will be made in the proper place. Figs. 2, 3, and 4 will serve as examples of what happens. They represent random samples of experiments performed so as to give

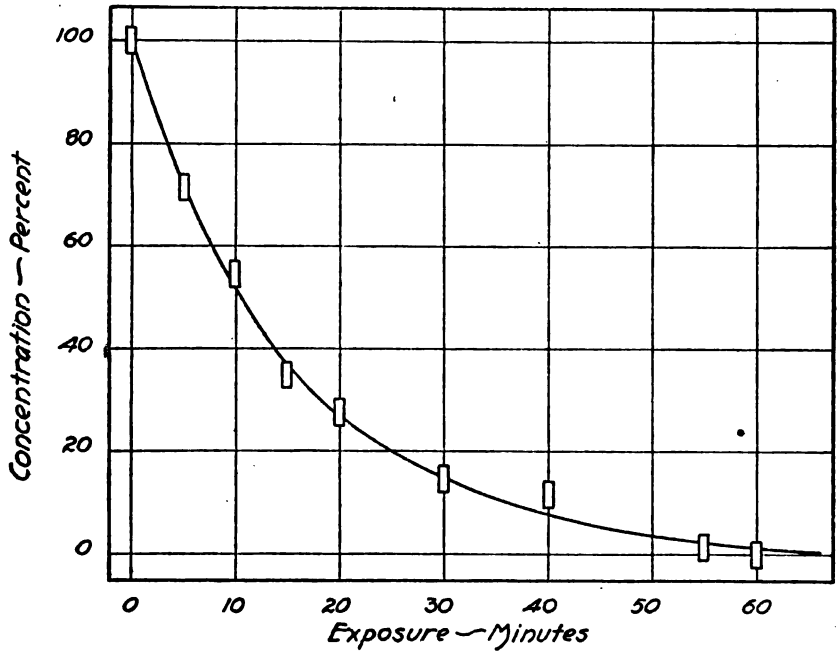

FIG. 3. Results of Experiment 42. Intensity, 28 meter candles; $k = 0.028$.

different velocities of decomposition, and are quite characteristic of all the other experiments.

In the figures the readings are plotted as rectangles, on the assumption of an error of 5 per cent in the determination of the concentration, and 1 minute in the control of the exposure. The curves are isotherms of a monomolecular reaction

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

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in which a is the original 100 per cent concentration, and $a-x$ the per cent of material still unbleached. It is apparent that the experimental results follow the curves pretty well.

To show this in the usual way Table I is given presenting the results of a fourth experiment. It will be observed that the values of the velocity constant k are fairly constant. This uniformity is particularly striking if two things are kept in mind: first, that the measurements are single determinations only, not averages; and, second,

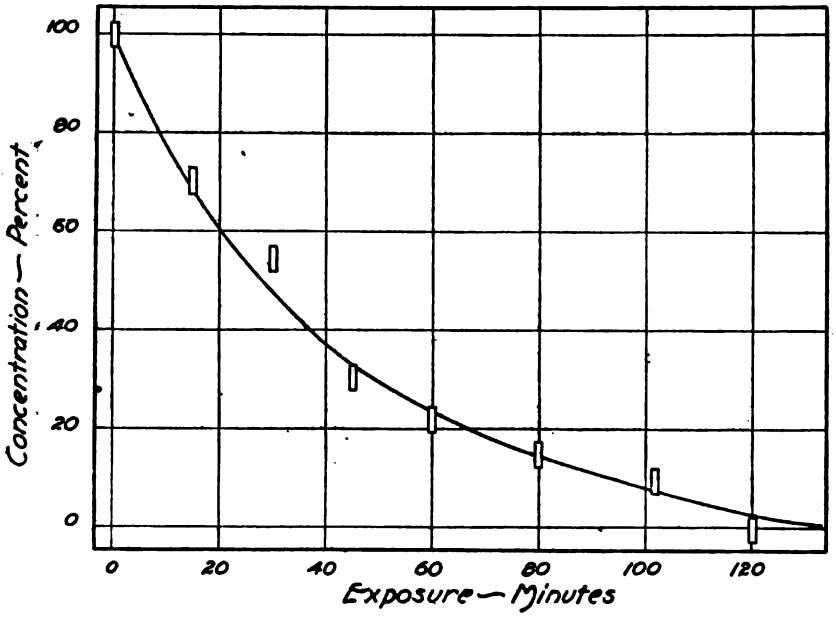

FIG. 4. Results of Experiment 56. Intensity, 12 meter candles; $k = 0.011$.

that they are read usually to the nearest 5 per cent concentration. For further comparison there are inserted the values calculated from the formula assuming the average value of k (= 0.030). The similarity between the observed and calculated values is obvious.

2. Each of the experiments so far considered represents the changes in a single tube of visual purple. The solution is exposed for an interval, its concentration determined, then exposed for a second interval, and the process repeated until no further change occurs. Such a

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mode of procedure assumes two things: one, that there is no period of induction in the photochemical action; and the other, that there is no after effect. If either of these is associated with the reaction, a different type of result,—qualitative perhaps, certainly quantitative, —is to be expected depending on whether the exposure is continuous or interrupted.

In order to settle this point several series of experiments were made in the following way. Seven or eight tubes of similar concentration and depth were prepared. One or two were run through in the ordinary way. The remaining tubes were exposed for a series of

TABLE I.

Experiment 85, Showing the Course of the Decomposition of Visual Purple by Light.

Time.	Concentration observed.	$k = \frac{1}{t} \log \frac{a}{a-x}$	Concentration calculated.
min.	*per cent*		*per cent*
0	100		
6	65	0.031	66
12	42	0.031	44
18	25	0.033	29
24	20	0.029	19
30	15	0.027	13
36	10	0.028	8
46	0		
Average.....		0.030	

increasing intervals; one for, say, 5 minutes, another for 8 minutes, another for 12 minutes, and so forth. In this way each solution was brought to a stage of decomposition by a continuous exposure as opposed to a series of intermittent exposures.

An example of such an experiment is given in Fig. 5. The black rectangles represent a single tube exposed intermittently in the ordinary way. The white rectangles each represent a separate tube of visual purple exposed continuously up to the point when its concentration was determined. The curve is again that of a monomolecular reaction, and it is apparent that it represents accurately the course of both types of experiment.

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In a few tests the procedure was slightly varied. Two sets of tubes were run. In the one the readings were made intermittently, there being about seven interruptions in the exposure. In the other, the readings were also made intermittently, but with only two or three interruptions in the exposure. The results in the two cases were the

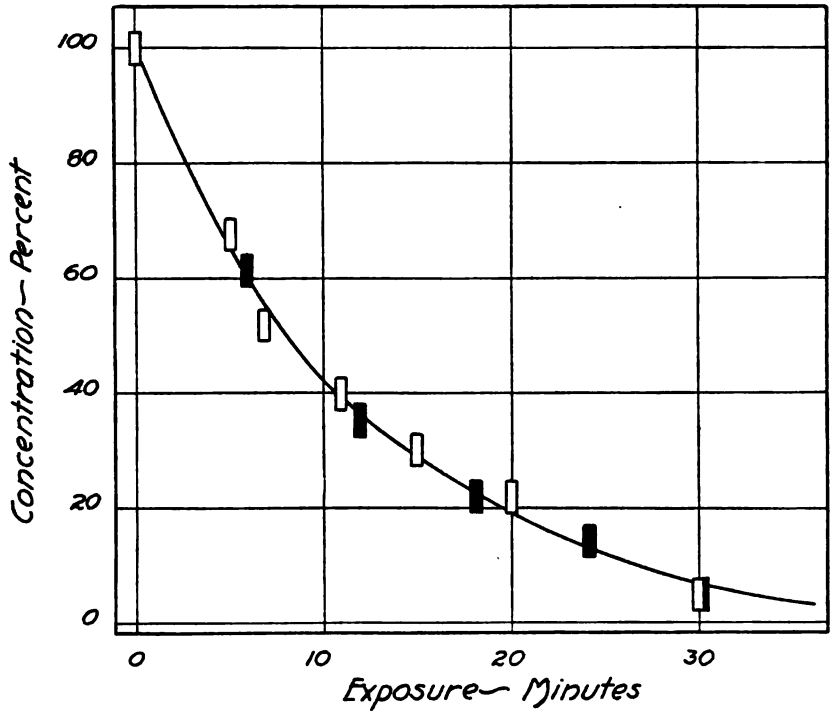

FIG. 5. A comparison of the effects of intermittent (black rectangles) and continuous (white rectangles) illumination on the velocity of decomposition. The black rectangles are from a single tube of visual purple. Each of the white rectangles represents a separate tube. Experiments 32 to 38 inclusive; intensity, 53 meter candles; $k = 0.037$.

same within the limits of the experimental error. It may therefore be safely concluded that there are no aberrant factors such as an induction period or an after effect operating in these experiments.

It will be remembered that these results are to be expected on the basis of Talbot's law for the fusion of successive light flashes. In such

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experiments the rate of intermittent illumination for the human eye is very much greater than those used here. The principle, however, is the same. Parker and Patten (1912–13) have recently shown that a continuous flow of light is a more efficient stimulus for the eye than an intermittent one (1,750 flashes per minute), the difference between the two being of the order of 5 per cent. It is not possible to compare these data with the present results on visual purple, because the experiments here reported do not admit of the judgment of differences of the order of 5 per cent in the magnitude of the velocity constant.

IV.

From the kinetics of visual purple decomposition it may be concluded that this photosensitive material is a chemical entity, and that its bleaching is probably represented by the destruction of a large molecule into smaller ones. In this respect it resembles the hypothetical sensitive substance postulated in the first section of this paper.

Too much reliance, however, must not be placed on the fact that the course of the reaction is monomolecular. Gross absorption and diffusion effects are, of course, eliminated by the thinness of the exposed solution; they therefore probably do not account for the monomolecular feature of the curve. But it is frequently true that the stoichiometric equation for a chemical reaction does not correspond to the equation obtained from kinetic data. In such cases (Lewis, 1918, p. 395) the stoichiometric equation turns out to be of a higher order. For example, it is conceivable that the bleaching of visual purple is a process of hydrolysis, in which case the stoichiometric equation may actually be bimolecular. However, because there is so much water present in the system, the reaction would proceed as if it were monomolecular, visual purple being the only component whose concentration changes to a measurable degree.

The equation

$$S \rightarrow P + A$$

for the hypothetical light reaction is also not to be taken dogmatically. It may well be

$$S + X \rightarrow P + A$$

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assuming that X is a substance whose concentration in the system is so great that it suffers no sensible change during the reaction. Further discussion of these matters will, however, be postponed to a time when more data will have been presented.

SUMMARY.

1. Visual purple solutions are prepared under such conditions that the bleaching reaction is irreversible.

2. A method is described for the colorimetric estimation of very small quantities of visual purple. By this means the kinetics of the bleaching reaction are investigated.

3. The results show that the course of the decomposition follows that of a monomolecular reaction, without any measurable period of induction or after effect.

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THE MECHANISM OF INJURY AND RECOVERY.

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(Received for publication, June 20, 1920.)

During the process of death the electrical conductivity of many tissues undergoes a change in electrical resistance by means of which the process may be followed with considerable precision. This may be utilized to measure injury and recovery.[1]

An illustration of this is seen in Fig. 1, which shows the gradual fall in resistance[2] of *Laminaria* placed in 0.52 M NaCl.[3] After an exposure of 5.2 minutes the resistance has fallen to 94.6 per cent of the resistance it had in sea water.[4] If the tissue is then replaced in sea water the resistance rises (uppermost dotted line) and returns to practically normal value. This rise of resistance may be spoken of as recovery.

[1] Attention has been called in a previous paper (Osterhout, W. J. V., *J. Biol. Chem.*, 1915, xxiii, 67) to the need of accurate determinations of toxicity and to the use of electrical measurements for this purpose.

[2] For convenience in making comparisons the death curves used in a former paper (Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533) are employed as standards. Since in the recovery experiments the death curves vary somewhat (depending on the temperature, the condition of the material, etc.), it is permissible for comparative purposes to make the death curves coincide with the standard in as far as this can be done by multiplying the abscissæ by the proper factor (which is always the same for all the abscissæ of any particular recovery curve). This has been done throughout the experiments: otherwise an accurate comparison of the recovery curves would be difficult. The corrections made are relatively small, since, in order to make the results comparable, all experiments showing a wide deviation from the standard curves were rejected. Since the deviations are chiefly due to differences in temperature the chief result of the corrections is to make all the reactions appear as if they had taken place at the same temperature.

[3] All the solutions had the same conductivity as sea water.

[4] For convenience all results are expressed as per cent of the original resistance.

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If, however, the exposure to the solution of NaCl lasts longer the tissue does not recover its normal resistance when returned to sea water. After prolonged exposure recovery is much less complete and as the tissue approaches the death point there seems to be no recovery when it is removed from the solution of NaCl and kept in sea water under normal conditions.

Fig. 1 shows that the recovery curves rise to certain definite levels and then run horizontally. If the conditions are favorable they may maintain this horizontal course for days; but if the control falls they also fall.

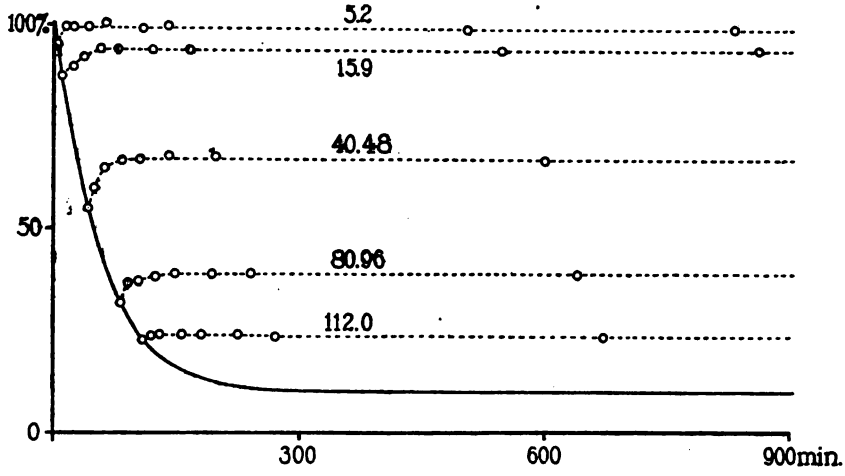

FIG. 1. Curves showing electrical resistance of *Laminaria agardhii* in NaCl, 0.52 M (unbroken line), and recovery in sea water (dotted lines). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the solution of NaCl.

Another aspect of recovery is illustrated by the results obtained in mixtures of NaCl and $CaCl_2$. Curve C in Fig. 2 shows the behavior of tissue placed in a solution containing 97.56 mols of NaCl to 2.44 of $CaCl_2$; its electrical resistance falling in 37.5 hours to 72.87 per cent of the original value in sea water. In a solution containing 85 mols of NaCl to 15 mols of $CaCl_2$ (Curve A) the resistance fell in the same time to practically the same point (72.47 per cent).

When these two lots of tissue were replaced in sea water they behaved differently. The resistance of the first lot rose to 78.2 per cent

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(Fig. 2, upper dotted line) but the resistance of the second fell (much more rapidly than if it had not been removed to sea water) and eventually became practically stationary at 38.1 per cent (Fig. 2, lower dotted line).

One method of explaining these facts is to assume[5] that the resistance is proportional to a substance *M* (contained within the cells), which is

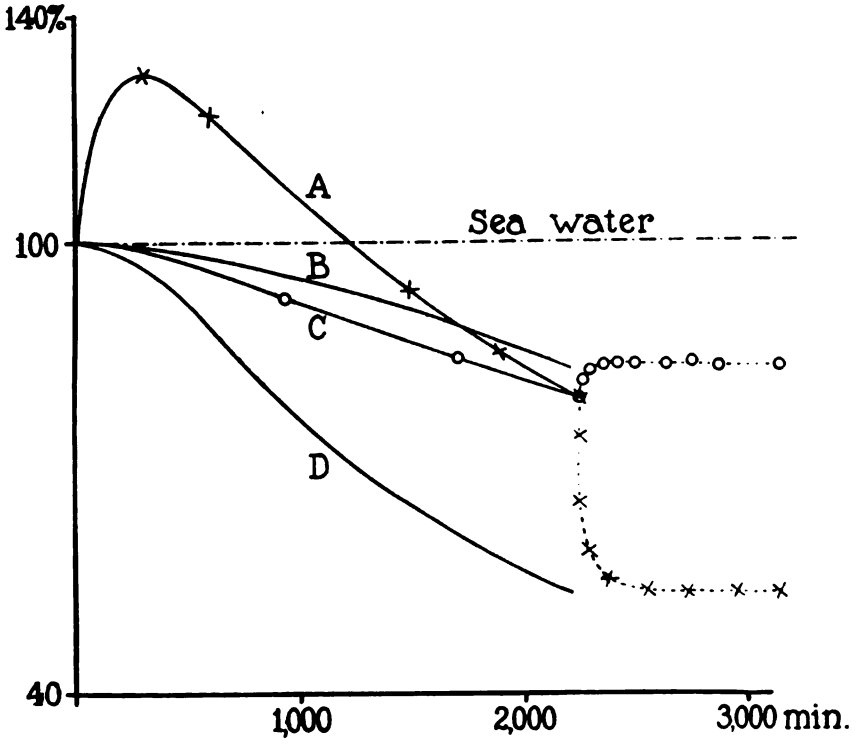

FIG. 2. Curves showing electrical resistance of *Laminaria agardhii* in a solution containing 97.56 mols of NaCl to 2.44 mols of $CaCl_2$ (Curve C) and in a solution containing 85 mols of NaCl to 15 mols of $CaCl_2$ (Curve A). The dotted lines show recovery in sea water. Curves B and D show the levels to which the resistance rises when the tissue recovers in sea water after exposure to these mixtures; their abscissæ denote the times of exposure. Curve B pertains to the first mixture (belonging with Curve C), while Curve D pertains to the second mixture (belonging with Curve A).

[5] *Cf.* Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

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formed and decomposed by a series of reactions according to the scheme

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$$

We assume that in sea water the amount of M remains constant because it is formed as rapidly as it is decomposed. When, however, it is transferred to a solution of NaCl (or to the first of the two mixtures mentioned above) the resistance falls, because M is decomposed more rapidly than it is formed. In the second mixture the resistance rises, because M is formed more rapidly than it is decomposed, but after a certain time the rate of formation decreases and the resistance falls steadily.

We suppose that when tissue is placed in the solution of NaCl (or in the mixtures) the reactions $O \rightarrow S \rightarrow A$ cease. In the solution of NaCl (and in the first mixture) the amount of M diminishes on account of the gradual exhaustion of A; in the second mixture the reaction $A \rightarrow M$ is so rapid that a temporary increase in the amount of M results, but this is inevitably followed by a decrease as the supply of A becomes exhausted.

When the tissue is replaced in sea water the reactions $O \rightarrow S \rightarrow A$ are supposed to recommence. The supply of A is therefore replenished and the resistance will return to the normal value, provided O is present in large amount and suffers practically no change during the experiment. But if it should diminish by one-half as the result of exposure to NaCl it could restore M to only half of its original value. We may therefore regard the level to which M is restored as an index of the condition of O. If we plot this level after various periods of exposure to the first mixture we get Curve B in Fig. 2 (and for the second mixture, Curve D).[6]

If we use the term recovery for the rise of resistance which occurs when tissue is transferred to sea water from certain solutions (such as the first mixture) there seems to be no good reason why it should not be applied to the fall of resistance which occurs when tissue is transferred from certain other solutions (such as the second mixture) to sea water. The amount of recovery after any given period of exposure

[6] A similar curve could be drawn for Fig. 1 but it has been omitted in order to avoid confusion.

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is equal to the vertical distance between Curves B and C, in the case of the first mixture, and between Curves A and D in the case of the second mixture.

If we use the term injury to denote loss of resistance, we may define temporary injury[7] as the loss which occurs in the solution and permanent injury as the loss which is observed after the tissue has been returned to sea water and the resistance has risen or fallen to a stationary condition. Curves B and D are therefore the curves of permanent injury.

It may be asked whether Curves B and D are better criteria of toxicity than Curves A and C. The question involves the definition of toxicity. Since this term is used in a variety of ways, it is desirable that it should always have a precise quantitative significance. In the present case it is evident that we need not only A and C but also B and D for a complete description of the facts. It seems possible that this may be generally true in the study of toxicity, although at present we may be unable to construct similar curves in many cases because suitable methods of measurement are lacking.

The fact that recovery is never complete except at the beginning (as shown by Curves B and D) might also be explained as due to the death of certain cells; for if some of the cells are killed by exposure to a solution of NaCl the complete recovery of the surviving cells cannot restore the resistance to its normal value. The chief objections to this explanation are that during the death process the cells seem to act alike and to die at about the same time; moreover this assumption does not lend itself readily to quantitative treatment. It does not seem to be necessary to discuss this point more fully at present; but it may be pointed out that this hypothesis would in no way invalidate the conception developed above, that an individual cell may lose part of its resistance and subsequently regain it, either partially or completely.

[7] Substances which cause increase of resistance commonly produce permanent injury; this is apparent when the tissues are replaced in sea water. It would therefore seem that any alteration of resistance (increase or decrease) may produce permanent injury if sufficiently prolonged. In spite of this it seems preferable to restrict the term temporary injury to the fall of resistance observed in toxic solutions without coining a new term to express the injurious action of rise of resistance.

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The fact that in the case of *Laminaria* recovery may be either partial or complete, according to circumstances, raises the question whether this is also true of other forms. It is certainly true of all the plants investigated by the writer, such as the green alga, *Ulva* (sea lettuce), the red alga, *Rhodymenia* (dulse), and the flowering plant, *Zostera* (eel grass). It seems to be also true of frog skin as far as the experiments of the writer have gone.[8] In physiological literature it seems to be generally assumed that when recovery occurs at all it is practically complete. It is evident that partial recovery might easily be overlooked except in cases where recovery can be measured with considerable accuracy, and it seems possible that further investigation may show that partial recovery is a general phenomenon.

If we accept the conclusions stated above we are obliged to look upon recovery in a somewhat different fashion from that which is customary. Recovery is usually regarded as due to the reversal of the reaction which produces injury. The conception of the writer is fundamentally different; it assumes that the reactions involved are irreversible (or practically so) and that injury and recovery differ only in the relative speed at which certain reactions take place.

Thus in the series of reactions $O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$, if the rate of $O \rightarrow S$ becomes slower than the normal, injury will occur, while a return to the normal rate will result in recovery. Injury could also be produced by increasing the rate of $M \rightarrow B$, or decreasing the rate of $S \rightarrow A$ or $A \rightarrow M$.

If life is dependent upon a series of reactions which normally proceed at rates bearing a definite relation to each other, it is clear that a disturbance of these rate-relations may have profound effects upon the organism, and may produce such diverse phenomena as stimulation, development, injury, and death. It is evident that such a disturbance might be produced by changes in temperature[9] (in case the temperature coefficients of the reactions differ) or by chemical agents. The same result might be brought about by physical means, especially where structural changes occur which alter the permeability of the plasma membrane or of internal structures (such as the nucleus and plastids) in such a way as to bring together substances which do not normally interact.[10]

[8] The recovery experiments on frog skin have been few in number and were devoted chiefly to the effects of anesthetics.

[9] *Cf.* Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxii, 23.

[10] Or which normally react to a lesser degree.

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INHIBITORY ACTION OF PARATYPHOID BACILLI ON THE FERMENTATION OF LACTOSE BY BACILLUS COLI. I.

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(Received for publication, July 8, 1920.)

In nature, organic matter is attacked by a large variety of bacteria and in its disintegration a number of species may take part. A knowledge of just how the species are associated in any given substrate or in what order they succeed one another in the process of decomposition might be of great value to the biochemist in supplying a point of approach hitherto neglected. There is evidence that there is some order in the mode of attack and that this order is conditioned by the nature of the medium and certain environmental factors, such as temperature, reaction, and oxygen supply. In the following pages one single detail of this general problem was examined under conditions which excluded all but two bacteria, one following the other.

If a fermentation tube containing fermented bouillon plus 1 per cent lactose be inoculated with some member of the paratyphoid group, the multiplication causes a heavy clouding or even turbidity in the open arm.

The growth in the closed arm depends on the degree to which the muscle sugar has been removed. If this is complete, no growth takes place. The clouding of the branch is therefore proportional to the amount of muscle sugar remaining. Titration shows little or no change of reaction in the closed arm, beyond what is due to a movement of fluid from open to closed arm. The fluid in the open arm becomes neutral and finally alkaline to phenolphthalein, the degree of alkalinity being a function of the multiplication which in turn is a function of the alkali-tolerance of the particular strain used. If after 4 to 6 days of incubation the same tube is reinoculated, this time with a true colon bacillus known to ferment lactose, one of two things may happen:

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1. The resulting fermentation takes the usual course as if no prior inoculation had been made; *i.e.*, the production of acid and gas goes on quantitatively as in a fresh tube.

2. The second organism multiplies actively, clouds the closed branch as usual, but no gas is produced. Titration shows that the usual amount of acid has been produced.

In order to determine whether the particular type of second or superadded fermentation is constant for the same organism, numerous tests have been made during the past 1½ years, with the result that with the same combination of cultures the same result follows.

Furthermore the various types of paratyphoid bacilli which happened to be on hand in the laboratory were tested to see just what grouping would result if this kind of superadded fermentation were used as a basis of classification. The results of this latter test are in many respects suggestive.

All true hog-cholera bacilli left the lactose medium uninfluenced. The fermentation of the second organism proceeded as in a fresh unused medium. Among the hog-cholera bacilli were those of widely different ages. The oldest one had been isolated by one of us early in 1886[1] and was described at that time as a membrane-producing variant. The most recent strain had been isolated by Dr. TenBroeck at Camp Upton late in 1918 (No. XVI).

Among cultures not isolated from swine, *Bacillus icteroides* Sanarelli acted like a true hog-cholera bacillus. That this organism may have been a true hog-cholera bacillus is made highly probable by the experiments of Reed and Carroll.[2] They found a complete identity of the two types in morphological, cultural, pathogenic, and agglutinative characters. *Bacillus icteroides* also produced fatal enteritis associated with necrosis of the mucosa of the large intestine in young pigs. One of these experiments was so carefully controlled that the, at that time, still unknown filterable virus of hog-cholera could hardly have come into consideration as a cause of the lesions. Moreover the autopsy notes do not suggest the filterable virus.

[1] Smith, T., *3rd Ann. Rep. Bureau Animal Industry, U. S. Dept. Agric.*, 1886, 622.

[2] Reed, W., and Carroll, J., *J. Exp. Med.*, 1900–01, v, 215.

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Of the cultures isolated from swine which failed to give the same reaction as the true hog-cholera bacillus was a non-motile organism isolated by Professor T. J. Burrill from the liver of a pig in 1897, and described briefly by one of us[3] in 1899. This strain could be differentiated from the true hog-cholera bacillus only through the absence of motility and a reduced virulence. *Bacillus coli* following this produced only a large bubble of gas in lactose bouillon.

Among the cultures tried which belong to the first group are Hog-cholera Nebraska (1886), Massachusetts (1895), Maryland (1898), Arkansas (1899), and Hog-cholera X, XI, XII, XIII, XIV, and XVI, each from a different outbreak; also *suipestifer* Voldagsen, and the non-gas-producing hog-cholera strain of M. Dorset. In several of the above strains mutants had appeared in the stock cultures. One of these produced a viscid growth, the other had lost the power of gas production in lactose. All these acted alike in not restraining gas production subsequently by *Bacillus coli*.

Among the paratyphoid and *enteriditis* strains in the collection the following came under the second group: paratyphoid strains recently isolated from four calves and one fetus by one of us; paratyphoid Strains 225, 232, 234, and 242; Schottmüller A and B; paratyphoid from a pigeon and a cow's brain, all received from other laboratories; Rat-typhus I, III, IV, and V; Swine-typhus I, II, III, IV, and V, Spermophile typhus I, Dog-typhus I, Guinea pig-typhus VII, VIII, Mouse-typhus I, II, *Bacillus enteriditis* 47, 204, Gaertner (Kral and Mt. Sinai), and swine *enteriditis*. The one exception noted thus far was marked paratyphoid (Longcope). Dr. TenBroeck[4] had previously examined this strain and found it culturally and serologically a hog-cholera bacillus. Its virulence, however, towards rabbits is very low as compared with true hog-cholera bacilli.

One culture of human typhoid tried thus far inhibited gas production and belongs therefore to the second group. A culture of paratyphoid bacilli from man which Hirschfeld has denominated Group C and which has been shown by TenBroeck[4] to belong culturally to the paratyphoid and serologically to the hog-cholera group, but with

[3] Smith, T., *Centr. Bakt., Ite Abt.*, 1899, xxv, 241.
[4] TenBroeck, C., *J. Exp. Med.*, 1920, xxxii, 33.

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a reduced virulence, acted like a true paratyphoid in inhibiting gas production.

The rather sharp distinction which this reaction makes between true hog-cholera bacilli and other paratyphoid strains made it seem desirable to test the several members of the dysentery group on hand. All strains tested, including those marked Shiga, Flexner, Hiss Y, Flexner-Harris, and Strong, acted like the hog-cholera group in that the second or *Bacillus coli* fermentation was not inhibited as to gas production. Similarly the group of paratyphoid-like bacilli attacking poultry (fowl typhoid and white diarrhea) did not inhibit gas production by *Bacillus coli*.

So far, to avoid confusion, the second fermentation has been restricted to two colon strains isolated from a calf. The strain of *Bacillus coli* used most constantly in the various experiments was isolated in December, 1918, from the liver of a calf (No. 302) affected with pneumonia and septic complications. It possesses motility to a certain degree, ferments dextrose and lactose with the accumulation of from 50 to 60 per cent gas in the closed arm of the fermentation tube, and produces a titratable acidity of 4 to 6 per cent of a normal solution. The gas formula H/CO_2 is of the usual type, the explosive portion being 2 to $2\frac{1}{2}$ times the CO_2 in volume. Saccharose is fermented more slowly and about 25 per cent gas accumulates. The second strain (No. 435) was isolated from the spleen of a very young calf affected with digestive derangements, called scours. It was in all cultural characters like the preceding.

Wherever gas production is referred to in the following pages, the accumulation of gas in the fermentation tube is meant. The acid produced is quoted in per cent of titratable acid in terms of a normal solution.

It is to be predicted that all races of *Bacillus coli* will not act alike since there are so many minor distinguishing morphological, cultural, fermentative, and serological features observed among them. Some preliminary tests with other colon bacilli have been made but the data are incomplete.

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Experiments to Determine the Nature of the Inhibition.

The Effect of Removing the Paratyphoid Bacilli.—The first suggestion to present itself was that the paratyphoid bacilli might have changed the lactose in some way. If so, in the bouillon deprived of the paratyphoid bacilli by filtration the colon bacilli should act differently than in fresh bouillon. It was found, however, that the complete removal of paratyphoid bacilli from the culture fluid by passing it through a Berkefeld filter restores gas production. The experiments were made by growing the paratyphoid bacilli in large centrifuge bottles and filtering after centrifuging. The filtrate was put into sterile fermentation tubes and inoculated with *Bacillus coli*. Table I gives some of the results.

TABLE I.

Strain of paratyphoid used.	Age of culture when centrifuged and filtered.	Results of inoculation with *B. coli*, Calf 302.	
		Gas.	Acid in open arm.
	days	*per cent*	
Calf 299.....	10	26.5	5.10
" 299.....	10	45.0	4.75
" 297.....	6	47.2	4.60
" 297.....	6	52.0	

The next step was to proceed as in the former experiment but to omit the filtration. The centrifuged fluid did not become entirely clear and when inoculated with *Bacillus coli* only a very little gas was produced. Apparently the presence of a very faint cloud of paratyphoid bacilli was sufficient to inhibit almost completely gas production by *Bacillus coli*. The hog-cholera control treated in the same way did not inhibit gas production (Table II).

In order to remove as far as possible all bacteria without subjecting the culture fluid to final filtration, the following procedure was carried out. The primary culture was conducted as in the foregoing experiment in large centrifuge bottles, holding 250 cc. and plugged with cotton wool. After an incubation of about 4 days sufficient to yield a maximum multiplication a certain quantity of sterile kaolin powder was added to the fluid, the bottles were closed with

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sterile rubber stoppers, and thoroughly shaken by hand. They were then centrifuged for about 30 minutes, at the end of which time the supernatant fluid was clear to the unaided eye in transmitted light. It was then transferred with sterile pipettes to sterile fermentation tubes and inoculated with *Bacillus coli*, Calf 302.

TABLE II.

Strain used.	Age of culture when treated.	Time of centrifugation.	Results of inoculation with *B. coli*, Calf 302.	
			Gas.	Acid in open arm.
	days	*min.*	*per cent*	
Paratyphoid, Calf 297.....	7	30	Bubble only.	3.80
" " 297.....	7	30	1–2	4.61
Hog-cholera XII.....	10	30	60	5.30

To determine whether the kaolin itself might add anything to the medium to influence the result various controls were introduced as shown in Table III.

TABLE III.

Effect of Centrifuging and Clearing First Culture with Kaolin on Second Culture.

Experiment.	Treatment of first culture. (Paratyphoid, Calf 299.)	Results of inoculation of second culture. (*B. coli*, Calf 302.)	
		Gas.	Reaction of open arm (acidity).
		per cent	
a.....	Sterilized kaolin added, bottle shaken and centrifuged 30 min., transferred to fermentation tubes.	65	4.56
b.....	Sterile control fluid, treated as under *a* before inoculating with *B. coli*.	93	4.55
c.....	Sterile control fluid without kaolin.	62	5.40
d.....	Fermented bouillon without lactose, shaken with kaolin.	0	0.27 (Open arm.)
			1.58 (Closed ")

Tube *a* shows that a complete clearing of the culture fluid from paratyphoid bacilli restores gas production by *Bacillus coli* and is equivalent to filtration. Tubes *b*, *c*, and *d* were to show whether kaolin modified the fermentation. The larger amount of gas produced in *b* indicates the presence of some favoring impurity on gas produc-

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tion, possibly a carbonate. Tube *c* checked the culture medium and Tube *d* proved the absence of fermentescible sugars in the kaolin.

Effect of Density of Suspension of Paratyphoid Bacilli.—In the foregoing tests the primary culture was usually 4 days old before the second inoculation was made. The mere presence of paratyphoid bacilli in the culture fluid might have some influence irrespective of any incubation period. Experiments were therefore undertaken to eliminate this period. The growth of paratyphoid bacilli on slanted agar was washed off with bouillon and suspended in lactose bouillon in fermentation tubes. The suspension was varied in density by using the growth of one, two, and four slants respectively. Immediately after the suspension was made a loopful of *Bacillus coli* was introduced from an agar culture and the tubes incubated. The gas production was not inhibited but it accumulated more slowly than in control tubes. After 7 days incubation 70, 68, and 66 per cent of gas had accumulated. The reaction of fluid in the bulb was 4.61, 4.96, and 4.91 per cent of a normal acid respectively. It should be stated that the density of the suspension of paratyphoid bacilli, even in the closed arm of the first tube, was much greater than that developing directly in lactose bouillon. No inhibition of gas production was thus produced by the mere presence of large numbers of living paratyphoid bacilli, both strains being introduced together.

In another experiment, the relation of the bacilli to the inhibition was put to a more rigorous test. A large centrifuge bottle containing lactose bouillon and inoculated with paratyphoid, Calf 297, was incubated for 6 days. One fermentation tube was then filled with the culture fluid and inoculated with *Bacillus coli*, Calf 302, and incubated as a control. Another lot from the same bottle was centrifuged until the supernatant fluid was only faintly clouded. This fluid was transferred to a second fermentation tube and inoculated with *Bacillus coli*. The thick bacterial sediment in the centrifuge tubes from 30 cc. of the culture fluid was transferred to 25 cc. of fresh lactose bouillon in a fermentation tube and inoculated with *Bacillus coli*. Over 50 per cent gas was promptly produced in the third tube. In the others a small bubble appeared partly derived from fine bubbles transferred with the culture fluid.

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The Effect of the Concentration of Lactose.—In the foregoing tests 1 per cent lactose was used throughout. It was thought, however, that less or more might affect the uniformity of results. Before taking up the experiment it became necessary to consider what minimum per cent of lactose was necessary for *Bacillus coli* to produce the maximum amount of gas. Earlier, repeated trials by one of us had shown that 0.5 per cent lactose yields about as much gas as 1 per cent or more. Table IV shows that the same result is obtained whether a concentration as low as 0.5 per cent or as high as 2 per cent lactose is used. Numerous tests of the gas formula in these experiments indicated no appreciable change in the relative amounts of H and CO_2. These tests have therefore been omitted from the tables.

TABLE IV.

Effect of Different Concentrations of Lactose on Secondary Fermentation by B. coli, Calf 302.

Primary culture.	Lactose.	Interval between primary and secondary cultures.	Gas produced.	Acid produced.	
				Open arm.	Closed arm.
	per cent	*days*	*per cent*		
Paratyphoid 297	0.5	6	0	5.0	5.0
Hog-cholera XII	0.5	6	57	5.2	
Paratyphoid 297	2.0	4	0	3.7	4.8
Hog-cholera XII	2.0	4	62	4.8	

The absence of lactose in the primary culture does not interfere with the establishment of inhibition. Cultures of paratyphoid bacilli in fermented bouillon without lactose will inhibit subsequent gas production by *Bacillus coli* when the latter and lactose are added at the same time after the primary culture has grown 4 or more days. To ensure the above results, there must be distinct clouding of the closed arm by the paratyphoid bacilli. Absence of multiplication due to a too thorough removal of muscle sugar fails to establish inhibition.

Effect of the Age of the Primary Culture.—Certain experiments had indicated that the age of the primary culture before inoculation with *Bacillus coli* was not an indifferent factor. Taking the combination

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paratyphoid, Calf 297, and *Bacillus coli*, Calf 302, *Bacillus coli* was inoculated at the same time with and hours after the paratyphoid bacillus to determine how long a growth period is required by the primary culture before it becomes completely inhibitory.

Table V shows that inhibition begins after 4 hours and is nearly complete at 14 hours with one lot of bouillon. With another it is not complete at 72 hours. This difference is probably due to the degree of removal of the last traces of muscle sugar during the prep-

TABLE V.

Effect of Age of Primary Culture on Inhibition of Gas Production.

Age of primary culture before second inoculation.	Amount of gas produced.	Acid produced in open arm.	Acid produced in closed arm.
	Bouillon 1,003.		
	per cent		
0 (Simultaneous.)	58	4.2	
2 hrs.	53	3.9	
4½ "	28	3.9	4.0
6½ "	24	4.2	3.9
8 "	20	3.8	4.2
10 "	11.5	4.4	4.2
12 "	10	4.9	4.2
14 "	5	4.6	4.9
	Bouillon 985.		
21 hrs.	13	6.0	4.7
2 days.	12.5	4.9	4.7
3 "	10	5.3	3.8
4 "	0	4.2	3.9

aration of the fermented (lactose) bouillon which controls the clouding of the closed arm.

It has been observed in certain experiments in which the second organism was introduced later than 4 days after the first, that in those cultures in which gas production was not inhibited, as in the hog-cholera group, the total quantity of gas produced was nevertheless not up to the usual amount. This observation led to the inference that perhaps here also the time element was a determining factor.

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Table VI gives the results of an experiment to clear up this question. Hog-cholera bacilli were introduced into a number of lactose tubes and kept different periods of time before *Bacillus coli* was introduced. Cultures incubated 19 days before the second inoculation inhibited gas production completely. Those incubated up to 6 days failed to inhibit. The rest produced variable amounts of gas.

The paratyphoid bacillus thus produces a condition in 2 to 4 days which the hog-cholera bacillus brings about in 18 days. It is interesting to note that this period roughly corresponds to the time

TABLE VI.

Effect of Age of Primary Culture on Inhibition of Gas Production. (Primary Culture, Hog-Cholera Bacillus XII; Secondary Culture, B. coli, Calf 302.)

Age of primary culture when *B. coli* inoculated.	Amount of gas produced.	Acid produced.	
		Open arm.	Closed arm.
	per cent		
0 (Control.)	65	5.0	4.9
3 days.	60	5.2	5.7
6 "	50	6.2	5.6
11 " *	5	5.9	5.6
11 "	32	4.6	5.2
14 "	7	4.8	5.5
18 "	0	4.8	6.0
19 " *	0	4.9	5.0

* Primary culture grown with about ½ of closed arm containing air. This was tipped out when *B. coli* was inoculated.

required by the hog-cholera bacillus to produce a translucency of milk when used as a culture medium. This translucency proceeds parallel with the increasing alkalinity of the medium. The different behavior of the paratyphoid and the hog-cholera bacilli is thus quantitative rather than qualitative, but the difference is such that at a certain time the phenomenon may be used as a qualitative test.

The gradual development of inhibition by the hog-cholera group is paralleled by a gradual loss of inhibition by the paratyphoid bacillus. This was demonstrated in the following manner. A culture of the paratyphoid bacillus, Calf 297, grown in lactose bouillon in a

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centrifuge bottle was transferred to fermentation tubes after 7, 24, and 32 days and then inoculated with *Bacillus coli*, Calf 302. The 7 day culture inhibited gas formation completely, the 24 day culture developed 44 per cent gas, the 32 day culture 35 per cent. The three tubes developed the usual amount of acid. In another experiment a culture of paratyphoid bacilli 22 days old yielded after inoculating with *Bacillus coli* 13 per cent gas. It is probable that the inhibition produced after 18 days by the hog-cholera bacillus is gradually lost later on, but no experiments have been made to test this assumption.

The Effect of Killing Paratyphoid Bacilli by Heat on Inhibition of Gas Production by Bacillus coli.

The results of the various experiments made thus far led to a study of the behavior of dead bacilli on gas production. Numerous experiments were carried out with a variety of controls in each but the results were not entirely concordant and pointed to some neglected factor. In all cases the exposure to temperatures which failed to kill the first culture failed to destroy inhibition. After the thermal death point had been reached the results became irregular, but the experiments all agreed in that inhibition was destroyed as the temperature rose and at 100°C. and above gas production was more or less completely restored. A careful analysis of the details of the experiments which are not reproduced here led to the hypothesis that mere death of the first culture is not sufficient to destroy inhibition but that there is another factor involved which disappears rapidly on exposure to high temperatures or gradually at lower incubator temperatures. To demonstrate the gradual disappearance of inhibition the experiments given in Table VII were made. Cultures of paratyphoid bacilli in lactose bouillon contained in large centrifuge bottles were exposed to 62°C. for 35 minutes. Subcultures were made at once and after 1 or more days of incubation to determine whether any bacteria had survived. The culture fluid was transferred to fermentation tubes at once and after the heated fluid had been incubated for 1 or more days. Subcultures were made at each transfer to determine sterility.

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TABLE VII.

Effect of Incubation of 5 Day Cultures of Paratyphoid Bacilli (Calf 297) Heated at 62°C. for 35 Minutes on B. coli (Calf 302).

No. of days in incubator.	Gas produced.	Acid in open arm.	Remarks.
	per cent		
0	6–7	4.87	Subculture sterile.
1	5	5.60	" "
3	60	5.10	" "
5	59	5.19	" "
0	4	4.97	" "
4	54	6.00	" "
7	56	5.18	" "

DISCUSSION AND SUMMARY.

Bacteria of the paratyphoid group may be divided into two classes according to the behavior of 4 day cultures in lactose bouillon after a second inoculation with certain types of *Bacillus coli*. One class includes all true hog-cholera bacilli, the other nearly all true paratyphoid and *enteriditis* types. Under the imposed conditions *Bacillus coli* produces the usual amount of gas in the presence of the first group. In the presence of the second no gas or only a bubble appears. The production of acid is not interfered with.

The significance of the inhibition was investigated in a variety of ways suggested by the particular hypothesis entertained at the time. Two main possibilities presented themselves; first, the direct association of the inhibition with living paratyphoid bacilli, and, second, the existence of a ferment or other product of growth as the inhibiting agent.

The theory that the living bacilli or those killed at the lowest possible temperature are responsible was favored by a number of experiments. Thus the complete removal of bacteria by filtration, or by centrifugation combined with the use of kaolin to produce a clear fluid restored gas production. The presence of a fine cloud of bacteria was sufficient to inhibit. On the other hand, the addition of large numbers of living bacteria from agar slants or from lactose bouillon after the requisite incubation period to fresh lactose bouillon

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failed to inhibit gas production when *Bacillus coli* was added simultaneously.

When the inhibiting culture was heated at 62°C. for 35 minutes to sterilize it, gas production was still largely inhibited. But it was restored when higher temperatures were used, completely at 100°C. and above. It was also gradually restored by exposing the heated culture to 37°C. for 3 or more days.

The presence of variable amounts of lactose, or even the complete absence of lactose did not interfere with the development of the inhibitory factor.

The activity of the inhibition factor presents itself in the form of a curve, beginning at 0 when both paratyphoid and colon bacilli are inoculated simultaneously and rising as *Bacillus coli* is inoculated at longer intervals from the paratyphoid bacilli. The maximum of inhibition is reached at about the 4th day; thereafter it remains at the same level for a few days and then gradually falls until it is lost within 3 or 4 weeks. The curve of the hog-cholera group is delayed in that the maximum inhibition is reached at the end of 3 weeks. These curves have not been accurately determined. Taking into consideration all the accumulated data the writers tentatively present the hypothesis that the inhibitory factor is some metabolic product of the paratyphoid bacillus, possibly an enzyme, which is destroyed at a temperature somewhat above the thermal death point of the bacilli and which more gradually disappears from incubated cultures. The substance fails to pass Berkefeld filters. It is carried down mechanically with substances clearing the culture fluid.

The experiments support current theories which hold that the acid-producing and gas-producing entities in cultures are distinct.

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COMPARATIVE STUDIES ON RESPIRATION.

XII. A Comparison of the Production of Carbon Dioxide by Penicillium and by a Solution of Dextrose and Hydrogen Peroxide.

By F. G. GUSTAFSON.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 11, 1920.)

The writer has shown[1] that the production of CO_2 by *Penicillium chrysogenum* is increased by acid and decreased by alkalies. The next step is to investigate the mechanism by which CO_2 is produced. For this purpose it seems desirable to try to construct an artificial system which would imitate the action of the fungus.

In the experiments with *Penicillium* the oxidizable substance was for the greater part dextrose, as the fungus was kept in a 0.5 per cent solution of that substance. A 0.5 per cent dextrose solution was therefore chosen as the basis of the artificial system. From a number of available oxidizing agents hydrogen peroxide was chosen as being the most promising. Dakin[2] states that of the various oxidizing agents hydrogen peroxide comes nearest to bringing about the same reactions *in vitro* as normally take place within the organism. An artificial system was accordingly made which consisted of a 0.5 per cent solution of dextrose plus hydrogen peroxide.[3]

The experiments were performed in essentially the same manner as those already described[1] except that the artificial system was substituted for the fungus. In each experiment the hydrogen ion concentration was kept constant during the whole experiment and the rate of production of CO_2 was measured before and after the addition

[1] Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 617.

[2] Dakin, H. D., Oxidations and reductions in the animal body, London, 1912.

[3] In these experiments "Dioxygen" was used, as it is nearly neutral and it is stated by the makers to contain only 0.04 per cent of substances other than water and hydrogen peroxide, no preservative being added.

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of hydrogen peroxide, while in the experiments with the fungus the hydrogen ion concentration was varied during the experiment. The results are nevertheless comparable in the two cases.

In the final experiments the same bottle of "Dioxygen" was used throughout, so that the results would be exactly comparable. It was found that the contents of two bottles which titrated the same with permanganate did not give the same results in respect to the amount of CO_2 produced. For this reason all the data here published were obtained by the use of hydrogen peroxide from the same bottle.

The time required to cause a standard change in the indicator tube, when the other tube contained 0.5 per cent dextrose but no hydrogen peroxide, was taken as 100 per cent. This change may have been caused by a leak in the apparatus or by the breaking down of the dextrose or by both. The source of the CO_2 is of no importance for the measurement. It is only necessary that in the absence of hydrogen peroxide the rate of production of CO_2 should be constant so that the rate found after the introduction of the hydrogen peroxide could be compared with it. For convenience the rate in the absence of hydrogen peroxide is called the normal rate; the rate after the addition of hydrogen peroxide is expressed in terms of the normal. This normal change was very slow and varied between 20 and 35 minutes.

In beginning an experiment the 0.5 per cent dextrose solution was made up to the desired hydrogen ion concentration; 60 cc. of this solution were put into the apparatus and the air was caused to circulate for 2 hours or more till all dissolved CO_2 had been pumped out. Several readings were then taken to get the normal rate of change. Then 8 cc. of hydrogen peroxide were added by opening the flask holding the dextrose solution; 3 minutes were allowed to elapse before the first reading was taken so as to allow a thorough mixing of the solution. In the first few minutes the production of CO_2 was very great, but as will be seen from Fig. 1, it fell off very rapidly in the solution having a pH of 1, while in the neutral solution the fall was not so rapid.

The experiments with the alkaline solutions were made with the direct method (without the apparatus for the circulation of air) as described in a previous paper.[1] Experiments were first made with neutral solutions. The dextrose solution was placed in a tube,

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hydrogen peroxide was then added, and 4 minutes were allowed to elapse. The time required to produce a definite[4] amount of CO_2 was then measured. The time it took to produce the same amount of CO_2 in the alkaline solution was then compared with the time in the neutral solution.[5] In Fig. 1, the comparison is expressed by taking

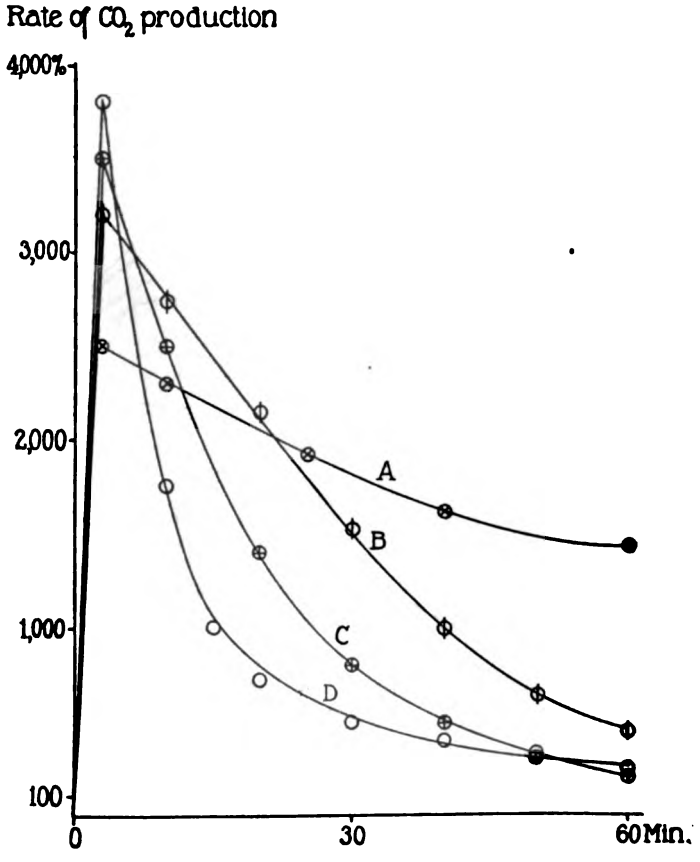

FIG. 1. Curves showing the rate of production of CO_2 by a solution of dextrose and hydrogen peroxide. Curve A shows the rate in a solution having a pH of 8.75, B in a solution of pH 7, C in a solution of pH 5, D in a solution of pH 1. The rate in the absence of hydrogen peroxide is taken as 100 per cent.

[4] This was ascertained by titrating with CO_2 as described in a former paper. (Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 617.)

[5] The experiments with alkaline and neutral solutions were carried out almost simultaneously.

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for the alkali curve a proportional part of the ordinate of the neutral curve obtained by the use of the apparatus. Thus if by the direct method the rate of production of CO_2 in the alkaline solution was two-thirds as great as in the neutral solution it was assumed that this would also be the case if the experiment could be performed in

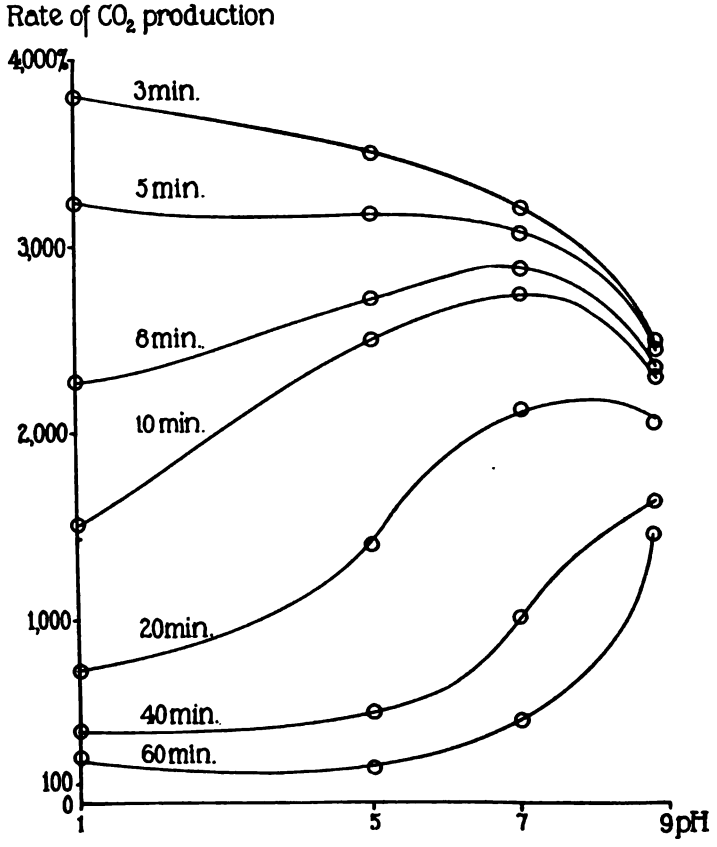

FIG. 2. Curves showing the rate of production of CO_2 by a solution of dextrose and hydrogen peroxide as related to the acidity of the solution.

the apparatus and accordingly the ordinate of the alkali curve in Fig. 1 was made equal to two-thirds the ordinate of the neutral curve.

From these experiments it was found that the production of CO_2 in the alkaline solution was at first below that at neutrality, but the fall in production was less rapid, and as will be seen from Figs. 1 and

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2 the rate was higher at the end of an hour in the alkaline solution than in the neutral solution. The decrease in production of CO_2 in the alkaline solution is not so great as that shown by the curve, because in taking more than one reading the solution had to be made alkaline again by adding more NaOH, and this produced a buffer action, which increased with every reading.

It is evident that during the first part of the experiment the production of CO_2 resembles that of the organism in being greater in an acid than in a neutral medium, but less in an alkaline medium than at neutrality. It should be noted that the increase of rate in acid followed by a decrease is also observed in the case of *Penicillium*.

The falling off of the rate in the latter part of the experiment might be due to a decrease in the amount of some substance. Evidently this is not the sugar; it is doubtful whether it is the hydrogen peroxide, since titration with permanganate as well as the catalase test shows that the hydrogen peroxide has hardly decreased at all by the time the rate has fallen off. It might possibly be an active form of hydrogen peroxide or a catalyzer. It is of course possible that the falling off may be due to the formation of an inhibitory substance.

According to Spoehr[6] there is no reaction between glucose and hydrogen peroxide in a neutral solution, but in a 0.5 M KOH solution 2 gm. of glucose had been completely used up at the end of 3 days, forming CO_2, formic acid, glycocollic acid, and α-hydroxymethyl-*d*-arabonic acid. As far as the writer is aware no work has been done with acid solutions.

It is probable that the reason why Spoehr did not notice any production of CO_2 in the neutral solution was because the amount is very small and the production lasts only a short time, while in the alkaline solutions the production continues for a longer time.

SUMMARY.

A neutral solution of dextrose and hydrogen peroxide acts like *Penicillium chrysogenum* in producing an increased amount of CO_2 upon the addition of acid, but not upon the addition of alkali.

[6] Spoehr, H. A., *Am. Chem. J.*, 1910, xliii, 227.

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DYNAMICS OF NERVE CELLS.

I. The Temperature Coefficient of the Neurogenic Rhythm of the Heart of Limulus polyphemus.

By WALTER E. GARREY.

(From the Physiological Laboratory of the Tulane University Medical School, New Orleans.)

(Received for publication, June 1, 1920.)

The temperature coefficient of the rate of heart beat has been determined by many investigators on various animals both vertebrate and invertebrate.[1] In all it was found that the coefficient for 10°C. was approximately 2 in the intermediate ranges of temperature, greater at lower temperatures, and somewhat less at high temperatures.

In none of these determinations has it been possible, owing to the character of the hearts worked with, to determine whether the results were due to an effect of temperature on the coefficient of ganglionic activity, of muscular activity, or of both.

It has therefore seemed of importance to determine whether variations of temperature, affecting ganglion cells alone, would give temperature coefficients of the same order of magnitude as those obtained on the whole heart.

A suitable preparation for this purpose is the excised heart of *Limulus polyphemus*, the beats of which have been shown by Carlson to be purely neurogenic.[2] Carlson has also described methods by which the ganglion can be separated from the muscle and independently subjected to variations of temperature while the muscle records the beats.

[1] For a general review of the literature of this subject see Kanitz, A., Temperatur und Lebensvorgänge, Berlin, 1915. Loeb, J., The Organism as a whole, from a physicochemical viewpoint, New York, 1916. Snyder, C. D., *Am. J. Physiol.*, 1911, xxviii, 167.

[2] Carlson, A. J., *Am. J. Physiol.*, 1905–06, xv, 220; also *Ergebn. Physiol.*, 1909, viii, 427.

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The ganglion of the heart of *Limulus polyphemus* occupies a median dorsal position on that organ; the nerve cells occupy a position over the posterior five or six segments of the heart and are connected with the anterior muscular segments by a median and two lateral nerves. This ganglion, which may be 10 or 15 cm. in length, can be dissected from the underlying tissue with ease and maintain functional connection with the muscle of the anterior segments.

In the experiments to be described a method was finally adopted in which a Dewar flask contained an immersion fluid, either sea water or *Limulus* blood serum. A system of glass tubes provided for renewal of the fluid at different temperatures which were recorded by a standard thermometer graduated to 0.1°C. Either the posterior segments of the whole heart, or the dissected ganglion, depended into the fluid through an opening in a paraffined cap. The two anterior segments were pinned to the cap, enclosed in a moist chamber, and kept at a constant temperature. The contractions of the anterior segments were graphically recorded and served as an index not only of the rate but of the effective strength of the impulses reaching the muscles. The study in its ramifications has extended to about 200 *Limulus* hearts.

Observations on the Whole Heart.

At Beaufort, N. C., the laboratory sea water was 27.7°C. and the average rate of 60 hearts was 23 beats per minute, while, at Woods Hole, the laboratory sea water was 20.1°C. and the average rate of 48 hearts was 11.6 per minute. A temperature coefficient of about 2.6 (Q_{10}) is obtained from these figures, calculating for the 10° interval. The individual hearts show wide variations in rate, and since the sea water of the two localities differs somewhat in concentration, this average coefficient for the whole heart can be considered only an approximation.

To illustrate the magnitude of the temperature coefficient of the whole heart for what may be considered a fairly normal range of temperature, ten experiments have been selected from our series—experiments in which it so happened that observations were made at exactly 15 and 25°C. The results are given in Table I. The experiments given in Table I show that the whole heart of *Limulus*, subjected to

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15 and 25°C. respectively, has rates giving an average temperature coefficient (Q_{10}) equal to 2.23. This indicates that the rate is determined by the alteration of a chemical process. The coefficient is quite in conformity with that reported for other rhythmic biological processes for this range of temperature.

TABLE I.

Rate per min. at		Temperature coefficient. (Q_{10})
15°C.	25°C.	
6	15	2.5
6.1	12	1.9
9	23	2.4
7.5	14.2	1.8
8	19	2.3
8.5	17	2.0
5	12	2.4
6.8	13.5	2.0
7.2	20	2.7
7	16	2.3
Average...7.1	16.17	2.23

In order to obtain a more comprehensive conception of the variation in the temperature coefficient of the whole heart subjected to different temperatures, summaries of some experiments are given below. The first shows the effect of cooling the heart to −2°C. and then progressively warming in a single but typical experiment.

Temperature range.	Temperature coefficient. (Q_{10})	Temperature range.	Temperature coefficient. (Q_{10})
°C.		°C.	
8 to −2	3.7	25 to 15	2.8
10 " 0	3.3	28 " 18	2.5
13 " 3	3.2	30 " 20	2.2
15 " 5	3.1	33 " 23	1.9
18 " 8	3.2	35 " 25	1.7
20 " 10	3.0	38 " 28	1.5
23 " 13	2.9		

The progressive change from a large coefficient at low temperatures to the smaller values at high temperatures is not always as uniform

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as illustrated in this experiment, but the coefficients at very low temperatures are usually larger than those recorded above.

Extremes of temperature may alter the value of temperature coefficients subsequently determined. It seems best in view of this fact to show separately the effects of depression and of elevation of the temperature starting with the normal temperature of the sea water. Each series thus is based on observations only on one side of the normal temperature, *i.e.* between it and a single limiting temperature, and thus the secondary effects of excessive heat or cold are avoided. In Table II the results of ten experiments are given in each of which the posterior seven segments of the whole heart with its ganglion were subjected to temperature variations.

TABLE II.

Temperature range.	Temperature coefficient (Q_{10}).						Temperature range.	Temperature coefficient (Q_{10}).			
	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.	Experiment 6.		Experiment 7.	Experiment 8.	Experiment 9.	Experiment 10.
°C.							°C.				
−2 to 8	6.0	4.6			7.6	7.2	20 to 30	2.3	2.5	1.8	
0 " 10	3.5	3.9	4.1	3.7	5.4	5.0	21 " 31	2.3	2.2		2.0
3 " 13		3.6		2.7			23 " 33		1.9	1.9	2.1
5 " 15	2.8	3.1	3.3	2.2	2.8	3.2	25 " 35	1.8	1.7		1.92
8 " 18		3.2		2.3	2.6		27 " 37				
10 " 20	2.5	2.8	3.0	2.0	2.66	3.0	28 " 38	1.6	1.5	1.7	1.67
11 " 21					2.5						

Observations on the Ganglion.

We may now compare the above results with those obtained by varying the temperature of the ganglion alone after dissecting it free from all adherent muscle tissue except the anterior segment. It would seem best for purposes of comparison with the results obtained above on the whole heart to observe the same precautions and to avoid the effects due to exposure of the ganglion to excessive heat or cold. The results tabulated in Tables III and IV are thus strictly comparable to those given in Table II since they show the simple effect of a progressive change in the temperature of the ganglion above or below the initial normal temperature of 20°C. In order to illus-

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TABLE III.

Effects of Cooling the Heart Ganglion Alone.

Experiment.	Temperature.	Rate per min.	Coefficient for 10°C.	
			Temperature range.	(Q_{10})
	°C.		°C.	
1	20	17	20 to 10	2.2
	17	13	17 " 7	2.9
	15	11.7	15 " 5	2.9
	10	6.2	10 " 0	4.3
	7	4.4	7 " −2	4.5
	5	4	5 " −2	7.2
	0	1.4		
	−2	1		
2	20	8.6	20 to 10	1.9
	15	6.4	15 " 10	2.1
	10	4.4	15 " 5	2.1
	5	3	10 " 0	2.9
	0	1.5	5 " 0	4.0
3	20	16	20 to 10	2.5
	15	10.5	15 " 5	2.8
	10	6.2	10 " 0	3.5
	8	5	8 "−2	6.0
	5	3.7	5 "−2	8.9
	0	1.7		
	−2	0.8		
4	21.6	11.8	21.6 to 11.6	2.8
	16.5	7.0	16.5 " 6.5	3.3
	11.6	4.2	11.6 " 1.6	5.6
	6.5	2.3		
	1.6	0.74		
5	19.7	12.6	19.7 to 10	2.3
	16	9	16 " 6	2.1
	12	6.6	12 " 2	2.2
	10	5.4	10 " 0	2.4
	6	4.3		
	2	3		
	0	2.2		

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trate the widest range of variation in experimental results, experiments on eight different animals were selected arbitrarily. As was expected the cardiac rate was slow when the ganglion was cooled and fast when it was warmed. In the lower ranges of temperature the temperature coefficient (Q_{10}) is well above 2 and at very low tempera-

TABLE IV.

Effects of Warming the Heart Ganglion Alone.

Experiment.	Temperature.	Rate per min.	Coefficient for 10°C.	
			Temperature range.	(Q_{10})
	°C.		°C.	
6	20	8	20 to 30	2.5
	22.5	12	22.5 " 32.5	2.1
	25	14	25 " 35	2.0
	28	20	28 " 38	1.6
	30	23		
	32.5	26		
	35	28		
	38	32		
7	20	12	25 to 30	2.2
	22	15	22 " 32	2.0
	25	17.5	25 " 35	1.9
	27	20	27 " 37	2.1
	30	27		
	32	31		
	35	33		
	37	35		
8	21	18.3	21 to 26	2.0
	26	26.8	21 " 31	1.8
	31	33	26 " 36	1.5
	36	40		

tures may be as large as 9. This places the process upon which the rate of the rhythmic ganglionic discharge depends unequivocally within the class of chemical reactions. The decrease in the coefficient at higher temperatures is the rule in both biological and chemical reactions and does not militate against this interpretation. The results in Tables III and IV are illustrated in Fig. 1.

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If we now compare the results obtained on the whole heart with those obtained when the ganglion alone is subjected to changes of temperature we are struck with the fact that there is not a single feature of the temperature coefficients which will serve to differen-

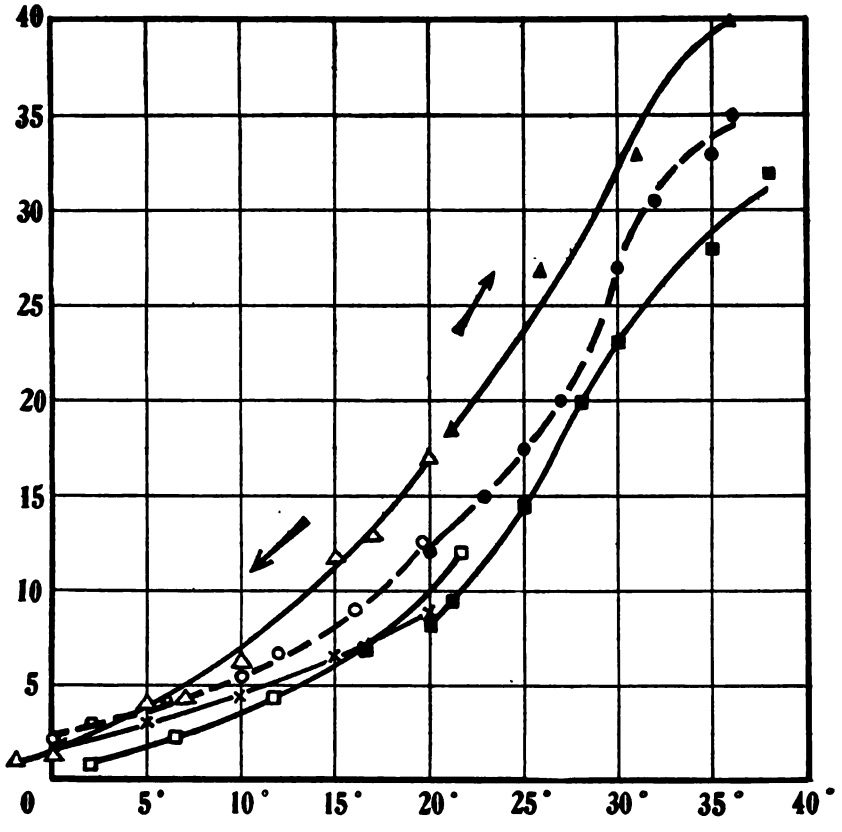

FIG. 1. Curves for the rates plotted against temperature. Data from Tables III and IV. △, ○, ×, and □ correspond to 1, 5, 2, and 4 respectively and show the effects of progressive cooling; ▲, ●, and ■ correspond to 8, 7, and 6 respectively.

tiate one from the other. The magnitude of the temperature coefficient and its variations are parallel in the two cases and we are justified in concluding that there is the same typical temperature coefficient for the rate of the heart beat of *Limulus* when the ganglion alone is

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subject to variations of temperature as when the whole heart is similarly treated. Furthermore chemical alteration in the ganglion determines the rate of heart beat.[3]

CONCLUSIONS.

In the case of the heart of *Limulus polyphemus* the same magnitude and variation of the temperature coefficient (Q_{10}) is obtained from the whole heart as from the ganglion alone. From the magnitude of the temperature coefficients and their variation with changes of temperature we may conclude that the rate of the heart beat is determined by alteration of chemical processes in the ganglion cells.

[3] The denervated heart muscle of *Limulus* was shown by Carlson to be without rhythmicity in its own blood or in sea water so that it is impossible to speak in connection with the tissue of a temperature coefficient which can have any bearing on the results communicated in this paper. The author has made this muscle rhythmic in M/2 NaCl and has been able to show that a rise of temperature of 10° may double the rate, but owing to the rapid incidence of toxic effects and to concomitant changes in rate, it is impossible to present reliable quantitative data at this time.

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DYNAMICS OF NERVE CELLS.

II. The Temperature Coefficients of Carbon Dioxide Production by the Heart Ganglion of Limulus polyphemus.*

By WALTER E. GARREY.

(From the Physiological Laboratory of the Tulane University Medical School, New Orleans.)

(Received for publication, July 24, 1920.)

That the rate of the heart beat of *Limulus polyphemus* is determined by the rate of chemical reactions within the nerve cells of the cardiac ganglion is shown by the magnitude of the temperature coefficient (Q_{10}) as determined by the author.[1] The differences in the temperature coefficient, the form of the rate curve, and the effects of extremes of temperature suggested the probability that the velocity of the chemical reaction was modified by a consecutive physical process with a different temperature coefficient. The formation of CO_2 in the nerve cells and its diffusion from them are suggested as processes analogous to those underlying the determination of the rate of impulse formation.

The following report shows that changes in temperature affect carbon dioxide formation by the ganglion cells in a manner which is quantitatively similar to the effect on the rate of the rhythmic contractions, thus establishing a basis for a hypothetical explanation to account for the different magnitude of the temperature coefficient at different ranges of temperature, and also for the change in the rate curve as affected by extremes of heat and cold.

* The experimental work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts. The author wishes to express his appreciation of the courtesy of the Director and staff of the Laboratory for placing the facilities for this work at his disposal.

[1] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 41.

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Method.

The rate of formation of even the small amount of carbon dioxide evolved from ganglia, each weighing only 12 to 30 mg., is not difficult if one uses the indicator method of Sörensen[2] as modified for tissue work by Osterhout and his collaborators.[3] The method of Tashiro,[4] who demonstrated that the *Limulus* ganglion produces CO_2, did not admit of rapid variation of the temperature and was not adaptable to our purpose.

The indicator method was used with the technique and precautions described by Moore.[5] The velocity of the reaction at different temperatures is determined by finding the time required to change the hydrogen ion concentration of an immersion fluid from pH 7.7 to 7.4 using phenolsulfonephthalein as the indicator.[6]

Buffer must be avoided in the immersion fluid which was a balanced saline solution of the following composition: 100 parts of M/2 NaCl, 1.5 parts of M/2 KCl, and 2.2 parts of M/2 $CaCl_2$, to 1 liter of which 15 cc. of 0.01 per cent phenolsulfonephthalein were added. The desired initial alkalinity (pH 7.7) was obtained by adding the requisite amount of NaOH.

The color comparisons were made in "Pyrex" glass tintometer tubes of about 4 cc. capacity, all of the same diameter, and filled with solution to a 3 cc. graduation mark. The tubes were stoppered with corks impregnated before each experiment with neutral, boiling paraffin or with rubber stoppers boiled in distilled water, paraffin-coated, and assuredly free from acid. The tubes and fluid were brought to the desired temperature before the ganglion was introduced and then kept at a constant temperature in large vessels of water. At the low temperatures it sometimes became necessary to adjust the tempera-

[2] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131; *Ergebn. Physiol.*, 1912, xii, 393.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171. Gustafson, F. G., *J. Gen. Physiol.*, 1918–19, i, 181. Brooks, M. M., *J. Gen. Physiol.*, 1918–19, i, 193. Thomas, H. S., *J. Gen. Physiol.*, 1918–19, i, 203. Irwin, M., *J. Gen. Physiol.*, 1918–19, i, 209.

[4] Tashiro, S., A chemical sign of life, Chicago, 1917.

[5] Moore, A. R., *J. Gen. Physiol.*, 1918–19, i, 613.

[6] The author is indebted to Prof. A. R. Moore who cooperated in the initial experiments of this investigation.

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ture by the addition of cold water or ice to the large containers but the variations in temperature were slight and negligible.

With scrupulous care to avoid injury, the ganglion was dissected from the heart and freed of all adventitious tissue, rapidly weighed, and immersed in the fluid (pH 7.7) to wash away all possible trace of acid and buffer. It was then placed in the tintometer tube at the desired temperature and the time required to assume the tint (pH 7.4) was determined with a stop-watch.

Results.

The results obtained in five experiments are presented in Table I. The relative velocity of CO_2 production is the reciprocal of the time

TABLE I.

Rate of Carbon Dioxide Production by Ganglia.

Temperature.	A Weight = 18 mg.		B Weight = 22 mg.		C Weight = 26 mg.		D Weight = 25 mg.		E Weight = 16 mg.	
	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}
°C.	*sec.*		*sec.*		*sec.*		*sec.*		*sec.*	
24	214		165		115		135		68	
14	393	1.8	345	2.1	319	1.9	445	3.3	282	4.0
4	1,800	4.5	1,245	3.6	778	2.4	1,920	4.3	1,480	5.2
14	247	7.4	290	4.3	290	2.6	645	3.0	360	4.1
24	75	3.3	111	2.6	144	2.0	180	3.5	108	3.3
34	37	2.0	65	1.7			110	1.6	59	1.8

in seconds required to change the tint of 3 cc. of solution from pH 7.7 to 7.4. The figures given are averages, usually of three readings, which in the present instance did not vary more than 3 per cent from the mean.

The results shown in Experiments A, B, and C (Table I) are typical. The temperature coefficient (Q_{10}) is equal to or greater than 2, in all except the highest range of temperature. The magnitude of the coefficient is of the same order as that previously found by Garrey[1] for the rate of the heart beat. Another similarity to the effects of temperature on rate of rhythm is to be found in the fact that it is usual that transient exposure to temperatures as low as 4°C. so affects

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the nerve cells that, after the temperature is again raised, the activities are pitched at a higher level than that maintained at this temperature prior to cooling. But while this is usual it is not an invariable result of cooling as is shown by the two experiments summarized in D and E (Table I).

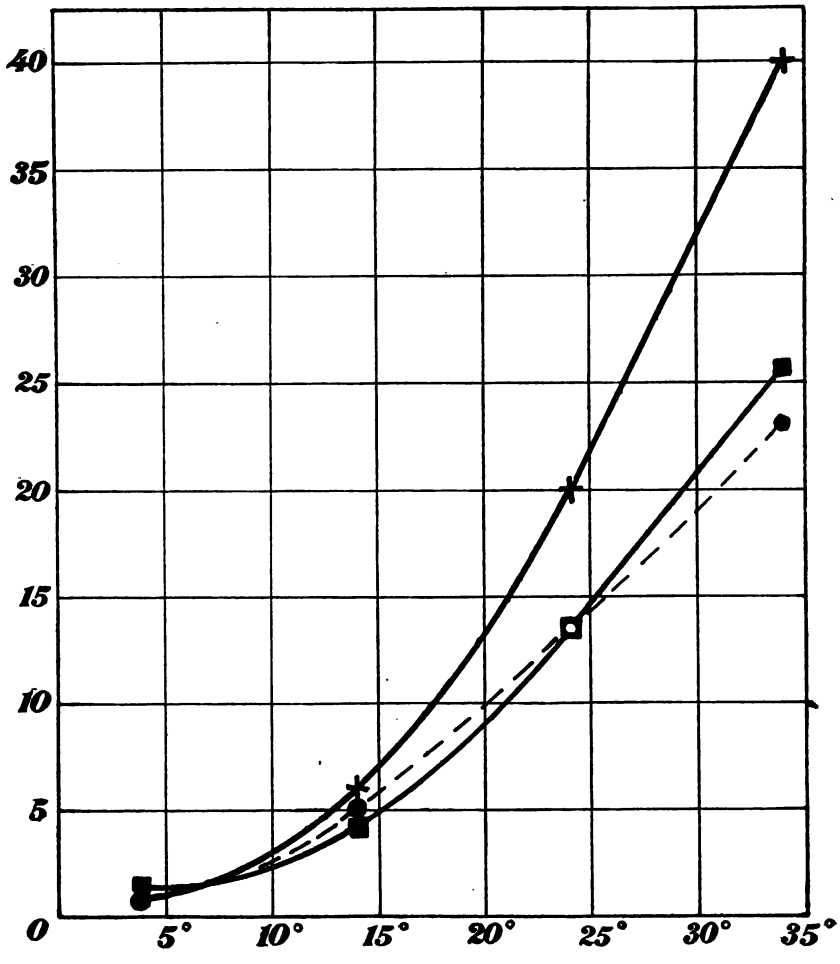

FIG. 1. Curves of the relative velocities of CO_2 production by *Limulus* heart ganglion at different temperatures. The data from Experiments A, B, and E, Table I. The reciprocal of time in seconds is multiplied by 1,500.

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The relative velocities with which the nerve cells form CO_2 at different temperatures are also shown graphically in Fig. 1. The curves are obtained from data in Experiments A, B, and E of Table I and are constructed by plotting the reciprocal of time in seconds multiplied by a constant (1,500). These curves of chemical reaction velocities, like those of rate of heart beat, are exponential and not linear functions of the temperature.

Certain exceptions to the rule that the temperature coefficients of heart rate were large at low temperatures and decreased with a rise of temperature have been reported by the author. We submit below data which indicate an exception which likewise applies to the formation of CO_2 by the ganglion. The ganglion in this case was the largest obtainable weighing 32 mg. from a *Limulus* weighing 8½ pounds.

Temperature.	Tint matched after.	Q_{10}
°C.	sec.	
27	155	
37	72	2.1
27	175	2.4
37	79	2.2
27	160	2.0
37	76	2.1
27	153	2.0
17	300	2.0
27	153	2.0

The above data show the usual temperature coefficient (Q_{10}) equal to about 2 for the range 17–27°C. but the coefficient for the upper range, 27–37°C., is uniformly larger than that usually found in our other experiments. It is also seen that after the first treatment of the ganglion at 37°C. and then cooling to 27°, the rate of CO_2 development is somewhat slower than at 27° prior to the heating. This is usual but it should also be noted that at the end of the experiment the rate at which the carbon dioxide was developed was identical to the initial rate at 27°C. It is evident from this result that even frequent subjection of the ganglion to 37° did not cause injury but produced effects which were entirely reversible in character. The constancy of the coefficients (Q_{10}) at different temperature levels in

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this case is suggestive of the results found by Loeb and Wasteneys[7] for oxidation processes in *Arbacia* eggs between 3 and 30°C.

It might be supposed from the results recorded in the preceding pages that CO_2 production is not proportional to the ganglionic mass since the rate at which carbon dioxide is produced is often very different for ganglia of equal weight, but it is also true that the rates at which hearts of equal size beat likewise show normally such variations. These facts merely mean that the rate of metabolic activity of different ganglia is different, for if we make the comparison with tissue from a single ganglion we find that the CO_2 development is proportional to the mass. In the following experiment showing this, the whole ganglion weighed 21 mg. Its rate of CO_2 development

TABLE II.

Relation of Ganglionic Mass to Rate of CO_2 Production.

Temperature.	Whole ganglion = 21 mg.		Anterior part = 10 mg.		Posterior part = 11 mg.	
	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}
°C.	*sec.*		*sec.*		*sec.*	
24	75		145		140	
14	160	2.1	340	2.3	325	2.3
4	510	3.1	1,200	3.5	1,095	3.3
14	150	3.4	330	3.6	275	4.0
24	60	2.5	150	2.2	120	2.3
34	35	1.8				

at different temperatures was determined and the ganglion then divided so that the posterior portion weighed 10 mg. and the anterior part 11 mg. After division, the parts were kept for a short time in neutral saline solution to avoid any complication from the acid which might have developed due to the injury; the rate of CO_2 development by each portion was then determined for the temperatures to which the whole ganglion had previously been subjected. The results are given in Table II and show conclusively that in a given ganglion the carbon dioxide production is proportional to the mass of ganglionic tissue.

[7] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxvi, 345.

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That the cardiac ganglia of different *Limuli* should show individual differences in the rate of carbon dioxide development is not surprising but the physiological significance of the metabolic variations is not so apparent. In our first paper[1] attention was directed to the fact that there were marked individual variations in the rate of the cardiac rhythm of different *Limuli* but that all showed temperature coefficients indicating the chemical character of the causative reaction. The present investigation was undertaken to determine whether carbon dioxide development was affected by temperature like the heart rate, and while it has been demonstrated that this is the case it has yet to be shown that, given two ganglia of equal weight but with different heart rates, the rate of carbon dioxide development is proportional

TABLE III.

Relation of Cardiac Rate to Development of CO_2.

Temperature.	A Weight of ganglion = 18.4 mg.		B Weight of ganglion = 17.7 mg.	
	Heart rate per min.	CO_2 development. Time.	Heart rate per min.	CO_2 development. Time.
°C.		sec.		sec.
22	16	105	8	185
12	7	240	3	495
Q_{10}.....	2.3	2.3	2.6	2.7

to the rate of the rhythm. To settle this point two *Limuli* of the same size were selected which showed heart rates of 8 and 16 per minute respectively at room temperature (22°C.). The hearts were excised and the rate of each at 12° was determined, after which the ganglia were dissected from the hearts and the rate of carbon dioxide development was then determined. The results, compared with the previous rates, are given in Table III.

The results of this experiment (Table III) leave no doubt that the rate of CO_2 development by the ganglia and the rate of cardiac rhythm run parallel courses.

All the experimental results herein reported thus show that the temperature coefficients of carbon dioxide production by the ganglia are of a magnitude entirely commensurate with those previously re-

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ported by Garrey for the rate of neurogenic rhythm of *Limulus* hearts. The data strongly support the view that the chemical process within the nerve cells which is responsible for the rate of the heart beat is one which is associated with the development and rate of diffusion of carbon dioxide.

SUMMARY.

1. It is possible to determine by the colorimetric method the rate of production of carbon dioxide by the cardiac ganglion of *Limulus*.

2. Carbon dioxide formation in the cardiac ganglion was found to run parallel to the rate of heart beat for different temperatures.

3. The conclusion seems justified that the rate of cardiac rhythm of *Limulus* depends upon a chemical reaction in the nerve cells of the cardiac ganglion and that this reaction is associated with the production of carbon dioxide since the rate of beat and the rate of CO_2 production are similarly affected by changes in temperature.

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ON THE RÔLE OF AN INTEGUMENTARY PIGMENT IN PHOTORECEPTION IN HOLOTHURIA.

By W. J. CROZIER.

(Contributions from the Bermuda Biological Station for Research, No. 117, and from the Hull Zoological Laboratory, University of Chicago, Chicago.)

(Received for publication, May 29, 1920.)

The experiment recorded in this note was undertaken in 1917, with a view to testing the notion (Crozier, 1914–15) that the fluorescent, greenish yellow, integumentary pigment characteristic of some members of the genus *Holothuria* is concerned with their photic sensitivity. A functional connection of this sort was suspected because the presence and relative amount of the pigment seemed to be correlated with the degrees of photic stimulation in three species of *Holothuria* studied at Bermuda; a pigment of similar nature seems to occur (Crozier, 1917) in the West Indian *Actinopyga agassizii* which, like these species of *Holothuria*, is negatively heliotropic. Dubois (1914), in his book, had earlier suggested a photosensitizing rôle for the fluorescent (dichromatic) substances occurring in various worms and echinoderms. It was expected that the stimulating power of sunlight transmitted by a screen of this pigment might be different from that of sunlight directly falling upon one of these holothurians. It is known that in photochemical action the effects of light are due to the absorbed wave-lengths. With the skin pigment of *Holothuria* there is pronounced absorption in the visible spectrum. Hence it was assumed that light deprived of these absorbable rays, by preliminary passage through a layer of the pigment, might perhaps be less effective for excitation. This expectation was not realized.

The species employed was *Holothuria captiva*, a form very sensitive to light, which was usually found under stones; in dark caves, however, it was found creeping freely over the walls, showing that its occurrence under stones along the shore is in all probability due to its photic irritability.

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The time required for the orientation of individuals 6.0 to 6.5 cm. long by sunlight reflected from a mirror, was found to be 6 to 8 minutes at 20°C.; the animals were at the beginning placed at right angles to the incident light. Other things constant, the speed of orientation varies inversely with the light intensity. The same individuals, and others in addition, were then in a similar manner exposed to sunlight which had passed through an alcoholic solution of the integumentary pigment of this species. A thickness of solution was used sufficient to produce apparently complete absorption of wave-lengths shorter than 530$\mu\mu$; the pigment of *Holothuria captiva* does not exhibit a band spectrum (*cf.* Crozier, 1915).

It was found that under the influence of the filtered sunlight, orientation required on the average 6 minutes—the same time as noted with the unfiltered light.

The photodynamic activity of eosin exhibits a peculiarity, discussed by Huber (1905) and by Clark (1918–19), which corresponds in certain respects with the apparent failure of a screen of *Holothuria* pigment to "protect" the animal to any degree from the stimulating action of light. The photodynamic action of eosin on rennin is found not to be materially lessened by the interposition of an eosin screen; hence the fluorescence of the photodynamic agent cannot be regarded as essentially concerned in any way with the production of the toxic effects. Clark (1918–19) has suggested that in such cases the essential photodynamic action (production of toxic materials) may depend upon wave-lengths for which the fluorescent substance is partially transparent.

Light which has passed through an adequate layer of *Holothuria* pigment will not excite fluorescence in a subsequent layer, hence the fluorescence must be due to wave-lengths readily absorbed. But light filtered in this way is not detectably weakened as a stimulating agent[1] for this animal. It must therefore be presumed that, if the pigment is in reality implicated in photoreception, light not readily absorbed by it is concerned in certain photochemical transformations differing from those which find expression in dichromaticity.

[1] Note that in this experiment the criterion of stimulating power has to do with the *rate of orientation* of the tested organisms—not with the rate of locomotion.

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STUDIES IN THE DYNAMICS OF HISTOGENESIS.

II. Tension of Differential Growth as a Stimulus to Myogenesis in the Esophagus.

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(Received for publication, April 3, 1920.)

INTRODUCTION.

Facts presented, for the first time, in a previous publication by the writer (1919–20) prove that the developing descending colon of the pig embryo possesses two zones of differential growth which by their interaction mutually influence each other during the formation period. The inner epithelial tube is the dominant, most active region of growth. It presents numerous mitotic figures which pursue a path cephalad, primarily, in the manner of a left-handed helix. The outer mesenchymal zone is less active in growth and early in development is composed of a uniform mass of undifferentiated cells.

The inner, rapidly growing, epithelial tube practically revolves due to the rapid spiral growth of its cells. With subsequent growth an apparent ring (this is qualified apparent for in reality a close spiral is formed) of smooth muscle myoblasts appears gradually near the periphery of the vortex.

This position taken by the inner, close spiral, smooth muscle coat at some distance from the epithelial tube is dependent upon an optimum tension. The attitude heretofore taken by embryologists eliminated the search for the underlying cause of the first formed, inner, muscle coat The theory of self-differentiation excluded interpretation as regards myogenesis. A certain mesenchymal cell, regardless of position, was considered as destined to become a myoblast.

This highly differentiated tissue is now considered to self-develop, for so called muscle-forming elements have been identified in the

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ovum (Conklin; Wilson). By the exclusion of this element in sectioning experiments the subsequent, positional, environmental relation is as much destroyed as the absence of the myoplasm. It may well be that the yellow pigmented zone is destined to assume a certain subsequent relation in development. This position may necessitate subjection to an optimum tensional stress stimulus due to the differential growth. The conclusion of self-differentiation is consequently unwarranted and too broad. All the work tending to support the generalization that muscle self-differentiates excludes the inner, environmental stimulus—the stretching or tensional stimulus of differential growth. Has the isolated myoplasm been cultured and found to form muscle? Only the affirmative answer to this question will warrant the assertion that muscles self-develop and then only providing the exclusion of surrounding germ plasm has been accomplished so as to exclude totally tensional stresses of differential growth of relational parts.

The last statement is made due to the fact that W. H. Lewis came to the conclusion that muscle self-differentiates from experiments in which transplantation of tissues around the otic capsule of tadpoles was performed. That musculature subsequently appears is not to be wondered at, for the potencies or actualities of differential growth were also misplaced with the transplant. If this piece remained viable it was bound to reveal subsequently the same tissues as in its normal location for the resultants of differential growth and the potential, mechanical stimulus due to space relation were left intact.

Consequently, the potencies of a blastomere are as much a function of its position as of its material substances. The material substances receive and react to the stimulus. The stimulus is a function of position. To elicit the response of mesenchymal cells in the formation of muscle tissue the proper optimum tensional stress stimulus must be applied. In tissue differentiation, therefore, the stimulus as well as the reception and response must be taken into consideration.

Tensional stresses are of various kinds and degrees. The quantity as well as the quality of stretching is important. The connective tissues are resultants of certain degrees of stresses. Muscular tissues, on the other hand, are responses to still different types of stresses.

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The submucosa interposed between the epithelial tube and the inner smooth muscle coat presents cells which react to a certain minimum of tensional stress. Just peripheral to the submucosa, muscular tissue is differentiated as a response to an optimum tensional stress.

It was observed by von Uexkull that in the nerve net of invertebrates the excitation flows into a stretched muscle. Extension, stretching, or elongation of a muscle cell precedes the desired effective contraction, therefore, as was inferred long ago by Hunter from observations on mammalian muscular action. It was also found by Cannon that there was a subliminal, an optimum, and a supermaximal tensional stimulus to elicit the response of the contractile tissue of the stomach in the normal stomachic movements. Evidently, an analogy is here found for the development of the musculature. There appears to be a subliminal, an optimum, and a supermaximal tension for stimulating the formation of contractile tissue. In normal development as well as in subsequent normal function the tensional stresses appear to be fundamentally involved.

Tension is due to a definite mechanical action. The formation of muscular tissue is due, therefore, to a definite active process, not a passive one as the term self-differentiation connotes. Loeb likewise concludes from his experiments on the gastrocnemius muscle of the frog that growth is an active, not a passive, affair as follows: "Activity, therefore, plays the same rôle in the growth of a muscle that the temperature plays in the growth of the seed."

The dominant growth of the epithelial tube and the resultant tension or stretching of the surrounding mesenchymal cells is strikingly exemplified throughout the digestive tract in its early stages of development. The object of the first part of this paper, therefore, is to demonstrate this interaction in the esophagus; furthermore, to interpret certain facts of subsequent torsional development of the alimentary tract in the light of the growth of the epithelial tube in the manner of a left-handed helix and the reaction of the mesenchyme to this epithelial growth. The origin of the spiral epithelial growth is also briefly considered.

To an advocate of the experimental sciences it is undoubtedly necessary that an actual experiment should be made showing that by gradual stretching of a cell, under the requisite circumstances, it

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is transformed into a muscle cell. To this end the writer is directing his attention. It must not be forgotten, however, that valuable suggestions pointing to a tensional stimulus as a factor in myogenesis is derived from a study of the origin of this tisue in a closely graded and advancing series of embryos. In the latter case direct observation reveals what is actually going on in nature's own laboratory.

Observations on the Early Development of the Esophagus of the Pig.

Hitherto, descriptions of esophageal development were written from a view-point regarding histogenesis as passive. No correlation of the developing epithelial tube and the surrounding mesenchyme has been presented. That one element could influence the other during the critical genetic steps has been overlooked. In order to comprehend clearly the development of the esophagus, or any formative structure for that matter, the active, dynamic point of view must be possessed by the observer and not the purely passive one.. The interaction and interdependence of integers united in a common structure must be considered together and not as isolated, non-related entities. It is from the dynamic aspect then that the following observations differ from previous accounts of esophageal genesis. It is also to be noted that certain developmental gaps are herein filled, thereby making the sequence of histogenetic events objectively evident.

Corresponding portions of the middle of the esophagus from an ascending series of embryos were selected for comparative study. This region was primarily chosen due to the fact that the lower cervical and upper thoracic portions have become narrowed due to the elongation of the esophagus concomitant with the descent of the stomach. The diminution in the diameter of this zone, leads to a more rapid revolution of the epithelial mitotic figures on account of the decrease in circumference of the epithelial tube. This more rapid rate of rotation causes a corresponding greater vortical agitation in the less active fluid like mesenchyme. The reacting maelstrom is consequently more clearly seen and the path of the activating mitotic figures more easily followed. The muscular rim bordering the mesenchymal vortex is also more clearly defined.

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The characteristic embryological facts will be presented in a closely graded series of pig embryos ranging from 9.5 to 24 mm. in length. It is during this short period that the genesis and growth of the inner, close spiral, smooth muscular coat is taking place. During the latter part of this period the outer elongated spiral or longitudinal muscular coat is becoming faintly indicated.

In a 9.5 mm. embryo (Fig. 1) the esophagus possesses a small epithelial tube composed of two to four rows of nuclei surrounding an oval or elliptical lumen. Enclosing the epithelial tube are found the undifferentiated mesenchymal cells. These are uniformly distributed in a spiral manner from the center to the periphery. The vortical arrangement of the mesenchyme together with growth of the esophagus in width and length depends upon the growth of the epithelial tube (Fig. 15).

The characters of the esophagus of pig embryos correspond closely to those found by F. T. Lewis and Johnson for human embryos of 8.4 to 16 mm. in length. Near the larynx it is crescentic in shape with the concavity of the crescent directed toward the trachea. This is a compression concavity due to tracheal growth. Caudad, the trachea first becomes round then transversely elliptical. In the upper thoracic region near the bifurcation of the trachea it is rounded but further caudad it merges with the stomach possessing a shape dorsoventrally elliptical.

The lumen is pervious and contains an albuminous liquid demonstrable in the living embryo. This liquid forms a reticular coagulum in fixed specimens (Figs. 1 to 6). The hydrodynamic influence of the liquid content found throughout the alimentary canal during mammalian development has been entirely neglected. In older embryos the accumulation of this intercellular fluid in isolated spaces which subsequently become confluent with the lumen is a mechanical aid in the separation and active moving apart of the cells resulting in enlargement of the lumen.

With further development the mesenchyme surrounding the esophageal tube becomes more discrete and defined from that enclosing the trachea. This is clearly seen in a 11.5 mm. embryo as shown in Fig. 2. The vortical arrangement of the cells is becoming more clearly marked. The mesenchymal cells show varying degrees of

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FIGS. 1 to 6. Development of the esophagus and trachea at corresponding levels through the lower cervical region of pig embryos ranging in length from 9.5 to 24 mm. (1) trachea surrounded by a nebular mass of undifferentiated mesenchyme; (2) esophagus, surrounded by a nebular mass of undifferentiated mesenchyme; lumen contains reticular coagulum; (3) left vagus nerve; (4) right vagus nerve; (5) inner close spiral muscle; (6) outer elongated spiral muscle; (7) ganglionic cells of myenteric plexus (Auerbach's plexus); (8) ganglionic cells of submucous plexus (Meissner's plexus); (9) intercellular vacuoles of esophageal epithelium; (10) mesenchyme and embryonic cartilage of the ventral aspect of the trachea.

FIG. 1. Transverse section through the lower cervical region of a 9.5 mm. pig embryo.

FIG. 2. Transverse section through the lower cervical region of an 11.5 mm. pig embryo.

FIG. 3. Transverse section through the lower cervical region of a 14 mm. pig embryo.

FIG. 4. Transverse section through the lower cervical region of a 15 mm. pig embryo.

FIG. 5. Transverse section through the lower cervical region of an 18 mm. pig embryo.

FIG. 6. Transverse section through the lower cervical region of a 24 mm. pig embryo.

The more rapid growth of the tracheal epithelial tube over that of the esophagus is to be especially noted. The mesenchyme around the trachea also grows at a more rapid rate than that around the esophagus. Consequently the less rapidly growing esophageal mesenchyme is thrown into a veritable vortex. This vortex represents a centrifugal reaction of the mesenchyme to the centripetal action of the esophageal epithelial tube. This tube is rapidly growing in the manner of a left-handed helix. At the periphery of the mesenchymal whirlpool the optimum tensional stimulus is presented resulting in the histogenesis of the inner, close spiral, smooth muscle coat. The outer muscle coat is faintly detected in the esophagus of a 24 mm. pig embryo.

The mesenchyme around the trachea, due to its rapid growth is compressed. This compression results in the formation of cartilage tissue. The embryonic cartilage is becoming evident in the trachea of a 24 mm. pig embryo (Fig. 6).

The reacting mesenchyme tends to converge between the trachea and the esophagus on the right and diverge on the left. The right vagus nerve appears to be drawn between the two tubes by this action.

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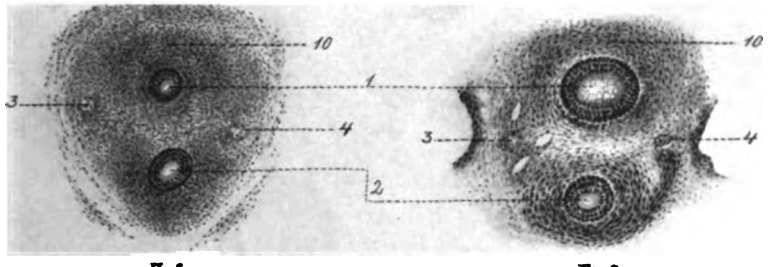

Fig. 1

Fig. 2

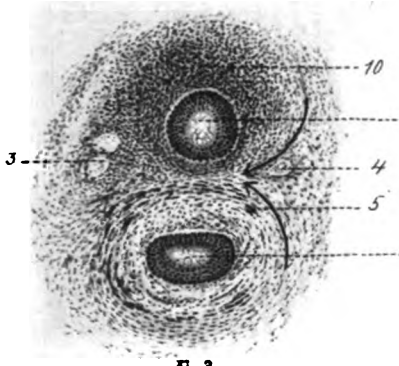

Fig. 3

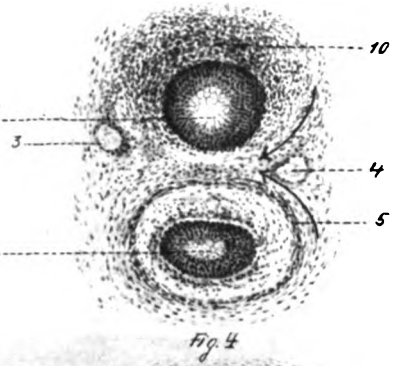

Fig. 4

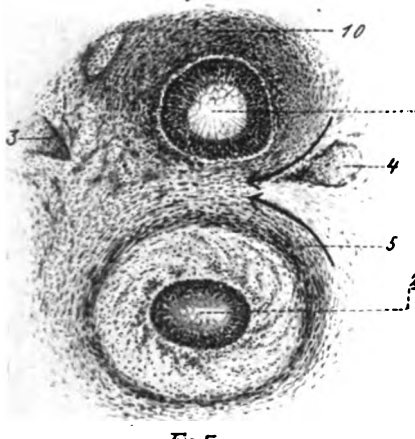

Fig. 5

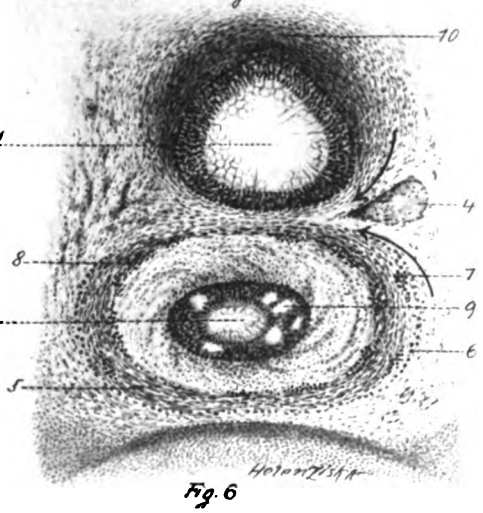

Fig. 6

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FIGS. 7 to 14. Cross-sections of the trachea and esophagus of a 9.5 mm. pig embryo. Note particularly that the mitotic figures of the trachea follow the path of a right-handed helix, whereas those of the esophagus follow the path of a left-handed helix. These two tubes, therefore, tend to rotate in opposite directions in development. This is comparable to the cylindrical rollers of a printing press. The mesenchymal vortices converge on the right side, represented by arrows, and diverge on the left. This is more evident in older specimens (see Fig. 6). (1) trachea; (2) esophagus; (3) proliferation bud of the trachea. These epithelial cells appear to be thrown off by an eruption and lost in the mesenchyme. At this location the basement membrane of the epithelial tube is absent. The rapid rate of growth of the tracheal epithelial tube probably causes this eruption by reacting centrifugal force.

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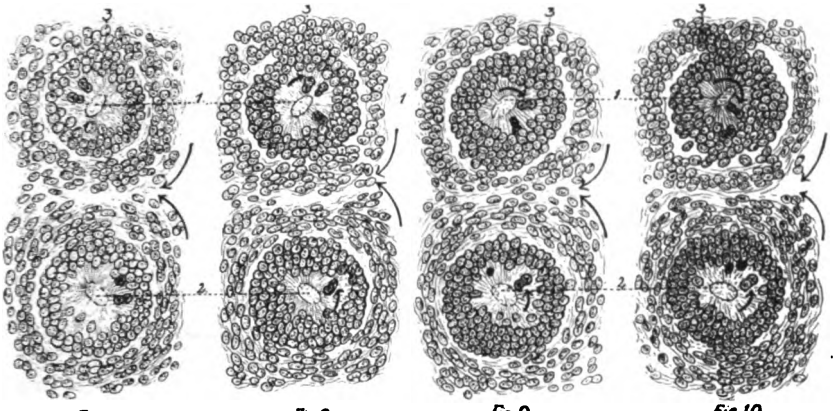

Fig. 7 Fig. 8 Fig. 9 Fig. 10

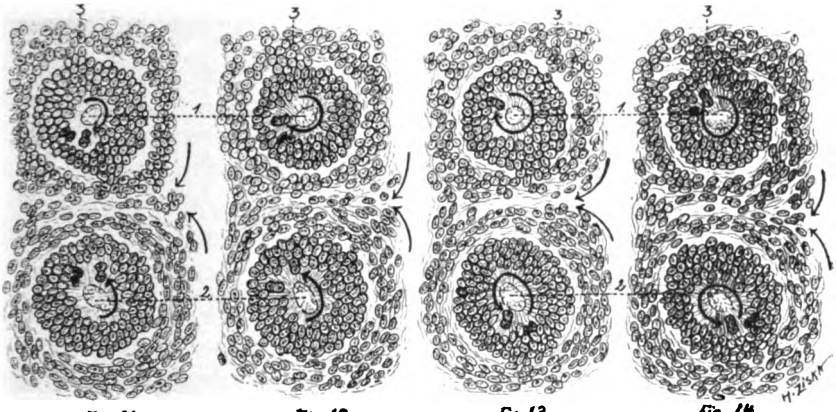

Fig. 11 Fig. 12 Fig. 13 Fig. 14

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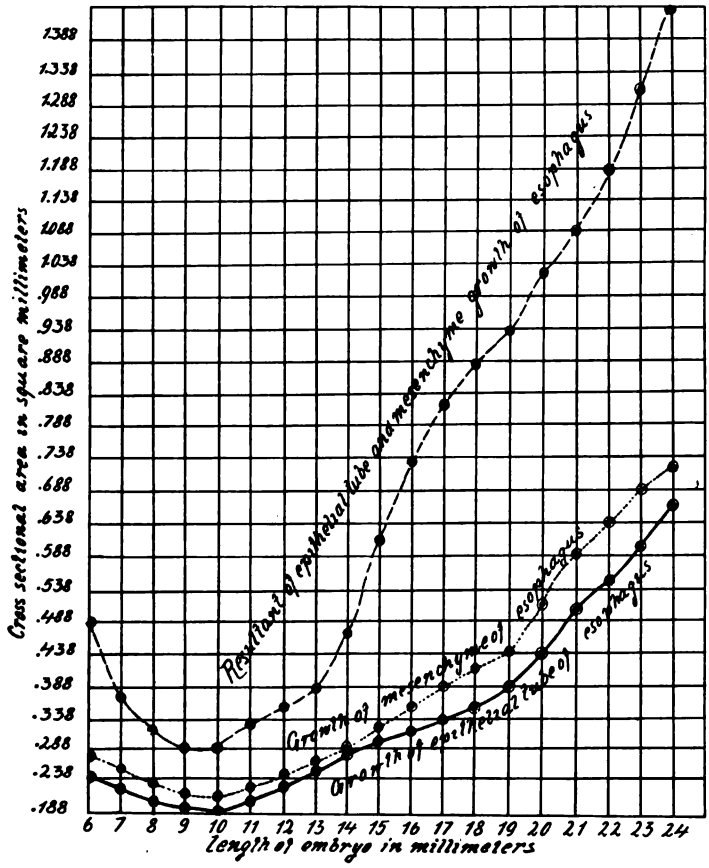

FIG. 15. Curves of differential growth of the epithelial tube and mesenchyme of the esophagus. Since there is no restricting peritoneal membrane in the lower cervical region of the esophagus the more rapid rate of growth of the epithelial tube over that of the mesenchyme is not as definite as that seen in the descending colon. The great extent of mesenchymal area is due to the centripetal force of the epithelial tube. The centrifugal reaction tends to throw the cells off tangentially. Consequently, the greater area of the mesenchyme is not due to an intrinsically more rapid rate of mitosis over that found in the epithelial tube but to the radial force (centrifugal force) of the mesenchyme in reaction to the centripetal force of the epithelial tube.

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elongation. Those at the periphery are more stretched than those centrally located. The nuclei are first elongated in the direction of the epithelial growth and shortly thereafter granules are found in the intercellular and intracellular cytoplasm. These granules are arranged in rows, likewise in the direction of the growth of the epithelial tube. Yet the distribution of the cells is fairly uniform throughout. No cell boundaries are presented. The entire region is a syncytium studded with nuclei surrounded by a narrow zone of granular endoplasm. The ectoplasm forms the connecting phase which characterizes the unity of organization found in the mesenchyme.

The epithelial tube, larger in the 11.5 mm. pig embryo than in the 9.5 mm. pig embryo (Fig. 1), possesses an increased number of cells. Numerous mitotic figures may be plotted in serial sections (Fig. 5). These form the path of a definite left-handed helix from above downward (Figs. 7 to 14).

The left-handed helix of the large intestine is also followed by the epithelial mitosis but from below upwards. The ileocecal valve is one point of convergence of the helicoidal growth of the epithelial tube. The increased growth in diameter of the large intestine with the path of mitosis directed cephalad meets the smaller diameter of the ileum with its mitotic path directed caudad. These are mechanical factors involved in the formation of the definite valve-like constrictors in the alimentary tract.

As development continues to the stage of a 14 mm. embryo there is a rapid increase in size of the epithelial tube. The lumen, although larger, is not correspondingly as large as the entire tube. The increase in size of the latter, therefore, is due to an increase in the number of cells crowding the lumen.

Concomitant with the rapid growth of the epithelial tube a beginning of formation of the apparent ring of stretched cells is found at the periphery of the mesenchymal vortex arranged in a tangential position. Various degrees in the elongation of the mesenchymal cells are found decreasing in intensity of stretching from without inwards.

On the outer side of the myoblastic ring the intercellular substance is drawn out into myofibrillæ. On the inner side of the ring, discrete granules are arranged in the same tangential direction as the elongated nuclei of the myoblasts. That the same force produces these

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results in the differentiation of muscle tissue is evident. These differential elements are elongated or arranged in the same direction around the ring. These structures represent the tensional reaction of the mesenchyme to the force of epithelial tubular growth.

The apparent annular formation of the mesenchymal cells at the periphery of the vortex is fairly established in an embryo 15 mm. in length (Fig. 4). At a distance the ring appears discrete but when examined with higher magnifications, there is revealed a gradual transition and blending of the degrees of stretching as the cells are examined from the periphery toward the central epithelial tube. The cells which have been subjected to the optimum degree of stretching for muscle origin at the periphery of the mesenchymal whirlpool react by forming myoblasts. Those more centrally placed have been evidently subjected to the optimum tension for the formation of embryonal, connective tissue. From direct observation it appears that the formation of a specific derivative from a pluripotent, mesenchymal cell is due to the fortuitous circumstance of position. Those cells at the periphery of the mesenchymal maelstrom form muscle, those toward the center form embryonal connective tissue.

The esophagus of an 18 mm. embryo (Fig. 5) shows still more clearly the inner, close spiral musculature, forming an apparent ring. Internal to the ring are found ganglionic clusters of the submucosa or Meissner's plexus; externally are found similar clusters and ramifications of the vagus nerve forming the myenteric or Auerbach's plexus. These are first detected in embryos 12 to 14 mm. in length.

The submucous, embryonal connective tissue interposed between the musculosa and epithelial tube contains numerous capillaries which by injection methods are demonstrated to pursue a spiral course. These capillaries form from discrete vesicles by confluences at the 15 mm. stage. With the development of the inner, close spiral musculature the connective tissue is still more definitely found to be arranged in spirals.

In the 24 mm. pig embryo (Fig. 6) the external elongated spiral or longitudinal muscle coat is becoming faintly indicated. This is more clearly seen in longitudinal than in cross-sections. This formation is taking place concomitant with the relative and absolute increase of the ratio of the length over that of the diameter of the esophagus.

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The precocious segregation of the esophageal epithelial cells would appear to be distinctly disadvantageous, for here a much larger proportion of each cell is in contact with others than in the looser texture of the mesenchyme. The intercellular vacuoles filled with fluid seen in the epithelium of the esophagus of a 24 mm. pig embryo (Fig. 6) is a provision for aiding excretion. These intercellular vacuoles are comparable to the periodic appearance of intercellular cavities observed by Kofoid in *Limax*.

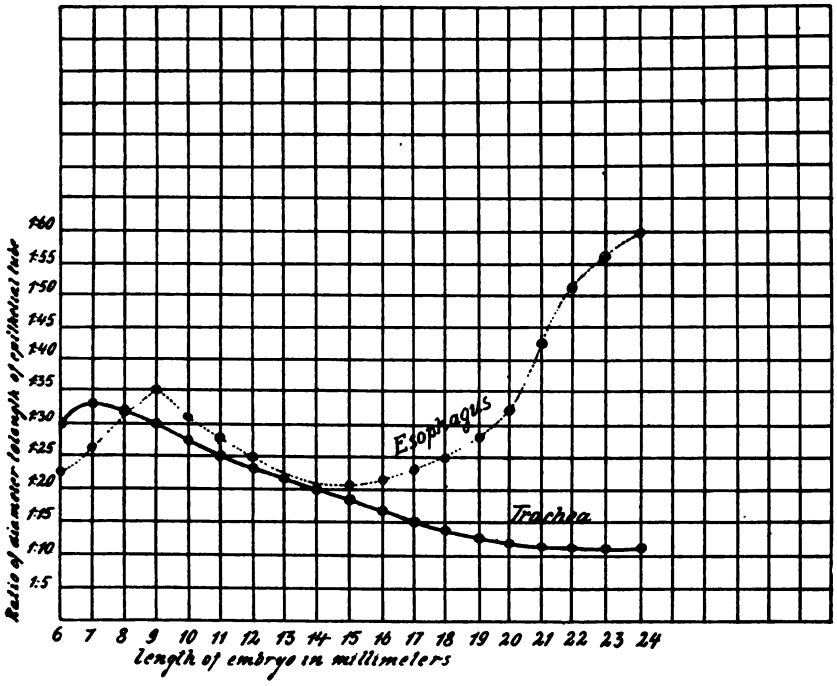

FIG. 16. Curves of ratio of diameter to length of the epithelial tubes of the esophagus and trachea. Note particularly that the esophageal tube grows relatively more rapidly in width in embryos 10 to 15 mm. in length, whereas, in embryos from 15 to 24 mm. in length the epithelial tube grows relatively more rapidly in length. In the former period the inner, close spiral, muscle coat is differentiating; toward the end of the latter the outer, elongated, spiral coat is being formed. The relatively greater growth in width than in length of the tracheal epithelial tube is objectively evident. This rapid growth is the efficient cause in compressing the tracheal mesenchyme, acting as the stimulus in the differentiation of the tracheal cartilage.

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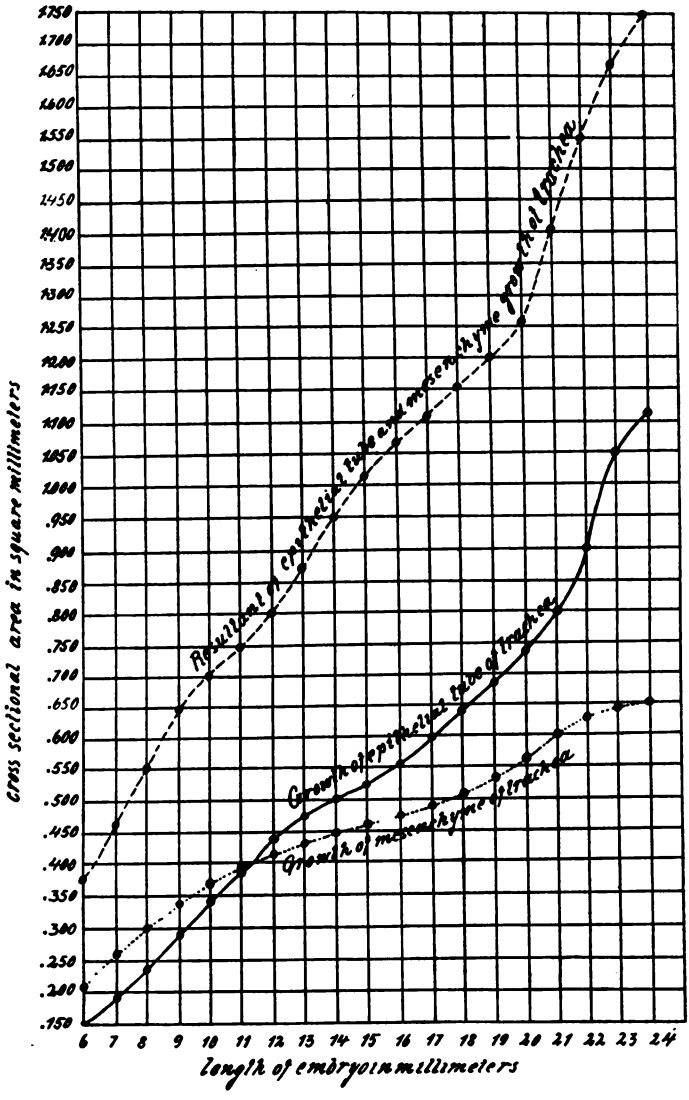

FIG. 17. Curves of differential growth of the tracheal epithelial tube and mesenchyme. The greater growth of the epithelial tube over that of the mesenchyme after the embryo has reached a length of 11 mm. is shown graphically. This rapid growth causes a progressive compression of the mesenchyme acting, therefore, as the efficient stimulus in the differentiation of cartilage tissue. The tracheal embryonic cartilage appears in embryos between 18 and 24 mm. in length. Note particularly that during this period the curve of epithelial tubular growth in area rapidly ascends.

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In the earlier stages the esophageal epithelial tube grows relatively more rapidly in diameter than in length (Figs. 16 and 17). In the 9.5 mm. embryo the esophageal epithelial tube measures 0.04 mm. in diameter and 1.3 mm. in length; in the 14 mm. embryo the diameter is 1 mm. and the length 2.1 mm. Therefore, the esophageal epithelial tube is growing relatively more rapidly in width than in length, from the 9.5 to 14 mm. embryonic stages.

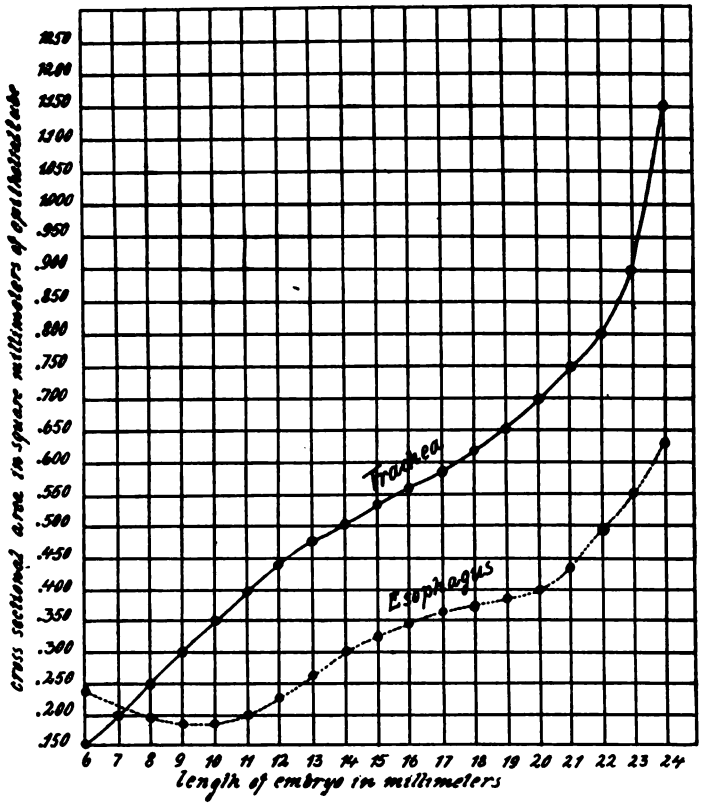

FIG. 18. Curves of differential growth in area of the tracheal and esophageal epithelial tubes. These curves show strikingly the more rapid growth in the same unit of time of the tracheal tube over that of the esophageal tube. Note that between embryos 6 to 10 mm. in length there is an absolute decrease in area of the tube. This decrease is concomitant with the rapid descent of the stomach. This decrease in area is due to stretching as the result of traction caused by the descent of the stomach. After the stage of the 10 mm. embryo, growth in area of the esophagus rapidly progresses.

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The reverse relation takes place, however, in embryos ranging in length from 14 to 24 mm. (Figs. 16 and 18). In the 24 mm. embryos the esophagus measures 9.1 mm. in length and 0.15 mm. in diameter. We see, therefore, that the length increases four times over that found in the 14 mm. embryo, whereas the diameter only increases one-half. These facts help us to coordinate intelligently, for it is now evident that the inner close spiral musculature is in the process of formation while the epithelial tube is growing relatively more rapidly in diameter and that the outer longitudinal muscle is incepted during the rapid elongation in length. At the 24 mm. stage the outer longitudinal muscle is faintly indicated. This outer coat is found, throughout development and in the mature state, less developed than the inner one.

Interpretation of the Primary Intestinal Torsion.

In embryos 5 to 10 mm. in length the alimentary tract undergoes a torsion from left to right through approximately 90°. The left aspect of the stomach becomes ventral and the right becomes dorsal. This involves the esophagus, the small intestine, and the large intestine. The result of this torsion in the large and the small intestines is a reacting looping. The colic fraction becomes ventrally placed to the small intestine. This loop whereby the colic portion is placed ventral to the small intestinal element is a constant fundamental relation in the Mammalia. No satisfactory explanation has been given for this characteristic fundament of form.

This characteristic loop is due to the reaction of the elastic mesenchyme, from left to right, reacting to the left-handed helicoidal growth action of the epithelial tube. With intestinal elongation an inevitable loop is produced in a characteristic manner. This is clearly demonstrable in Figs. 19 to 24. The intestino-colic flexure, therefore, is a mechanical torsional reaction of the mesenchyme. This right-handed helicoidal reaction is due to the left-handed helicoidal growth of the epithelial tube.

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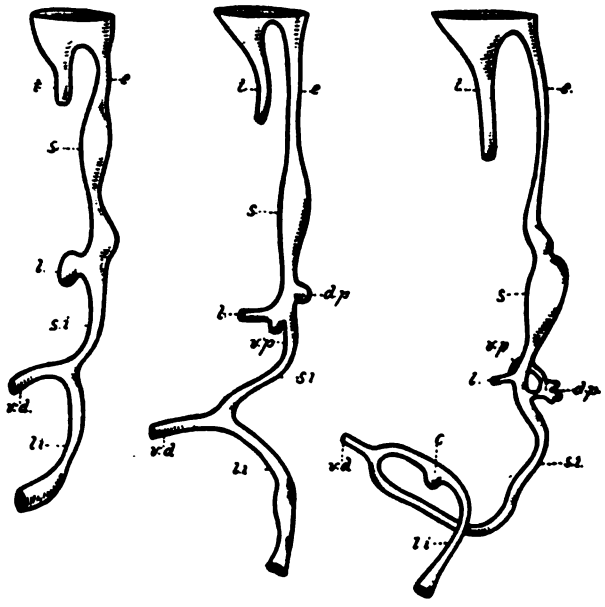

FIG. 19. FIG. 20. FIG. 21.

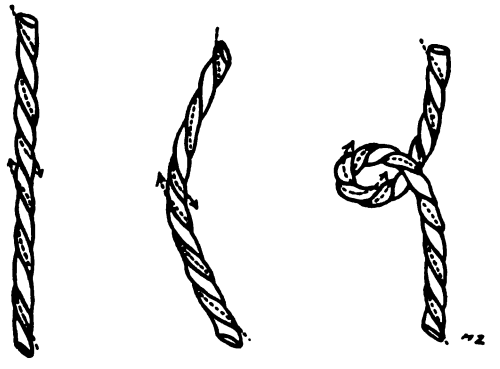

FIG. 22. FIG. 23. FIG. 24.

FIG. 19. Schema of digestive tract of a 6 mm. pig embryo.

FIG. 20. Schema of digestive tract of an 8 mm. pig embryo.

FIG. 21. Schema of digestive tract of a 10 mm. pig embryo.

FIG. 22. Schema of elastic band twisted as a left-handed spiral. This represents the mesenchyme of the straight digestive tract.

FIG. 23. Schema of ventral flexure of an elastic band; represents the ventral flexure of elongating tract of Fig. 20.

FIG. 24 Schema of inevitable ventral looping of caudal element over the cephalic element of an elastic band twisted as a left-handed spiral. This represents the reaction of the mesenchyme to the torsion produced by the spirally growing epithelial tube as schematized in Fig. 21. The loop tends to relieve the torsion to which the mesenchyme is subjected.

t, trachea; *e*, esophagus; *s*, stomach; *l*, liver; *d. p.*, dorsal pancreas; *v.p.*, ventral pancreas; *s.i.*, small intestine; *c.*, cecum; *v.d.*, vitelline duct.

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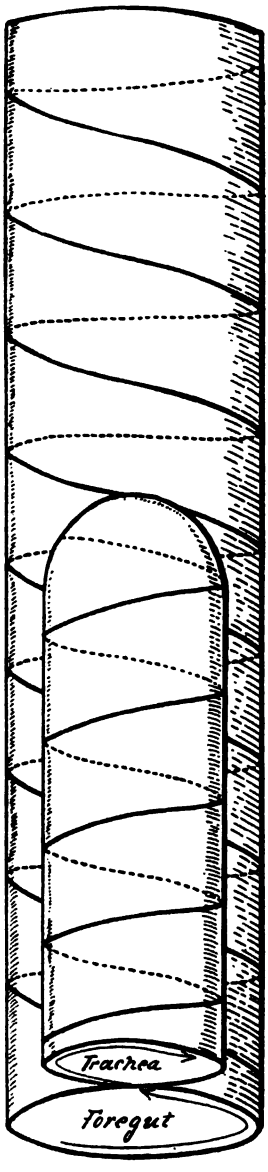

FIG. 25. Diagram of the tracheal bud from the ventral aspect of the foregut. The former presents mitotic figures following the path of a right-handed helix. The latter presents mitotic figures following the path of a left-handed helix. The tracheal or lung bud is an outgrowth from the ventral aspect of the cephalic aspect of the entodermal foregut. The cause of the mitosis pursuing a path of a right-handed helix in the trachea is due to the deflection of the left-handed, helicoidal, mitotic path of the foregut. This deflection occurs at the cephalic orifice of the tracheal epithelial tube where it arises from the floor of the entodermal tube of the foregut.

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This loop may be produced with a string or elastic band. With one end between the left thumb and index finger directed above and the lower element between the right thumb and index finger directed below we may exemplify the small and large intestines, respectively. By rotating the thumb and index finger clockwise, the string or band is wound as a left-handed helix. After this slight initial torsion, elongation of the intestine may be exemplified by approximating the left and right hand. A looping of the segment of the string in the right hand over that of the left is an inevitable reaction due to the direction of the torsion. A reverse looping may be produced by winding in the opposite direction or by excessive torsion in the previous direction.

The direction of epithelial growth is counterclockwise or as a left-handed helix. The elastic mesenchyme is drawn out in a corresponding direction. The resultant reaction of the mesenchyme is in the reverse direction from left to right. This is an inevitable reaction of the mesenchyme to the active torsion produced by the dominant epithelial growth. The torsion of the digestive tube, therefore, is a mesenchymal reaction to the torsional action of growth of the epithelial tube.

Situs Inversus Viscerum.

The normal asymmetry of the abdominal viscera as well as the position of the gut is dependent upon the action of the spiral growth of the epithelial tube and the resultant reaction of the mesenchyme. The left-handed helicoidal growth of the epithelium causes a corresponding arrangement of the cells of the less actively growing mesenchyme. The cells of the latter are elongated or stretched. Subsequently these mesenchymal cells react in the opposite direction from left to right. This causes the 90° rotation of the gut in a clockwise direction. The ventral position of the liver bud prior to rotation is now directed to the right comparable to the 90° displacement dorsally of the right vagus nerve after rotation is completed.

Pressler produced experimentaly *situs inversus* in *Bombinator*. While the embryo was in the neurula stage Pressler exsected a quadrilateral piece of the medullary plate plus a part of the dorsal surface of the primitive gut. This piece was then replaced in the reversed

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position so that the cephalic end was directed caudad and the caudal end was directed cephalad. Tadpoles were reared from these experimental embryos and in many cases a complete *situs inversus viscerum* was presented.

The transposition of the viscera is due to the fact that the reversed segment presents a screw-like growth action of the epithelium opposite to that normally present. The mesenchymal cells form a right-handed spiral arrangement and in reacting to stretching cause a 90° rotation of the gut from right to left, or counterclockwise. This reversed rotation due to the torsional reaction of the mesenchyme would cause the ventrally placed liver bud to be directed to the left instead of to the right.

An excellent discussion of the views of Wilder, Bateson, and Newman as regards mirror imaging and symmetry reversal in monsters will be found in a paper by Morrill. That Morrill had a clear-cut perception of the location in which solution of the problem of normal asymmetry of the viscera was to be found is seen in the following statement: "the factors controlling asymmetry are located in the primitive gut and become operative before the liver bud has developed."

Crampton suggested that asymmetry in the viscera may depend upon spiral cleavage during the first stages of development. This rested on the fact that the majority of snails possess a dextral shell associated with a right-handed spiral cleavage. Certain snails have a sinistral shell and reversed asymmetry in the viscera. This reversal of the viscera was associated with a left-handed spiral cleavage.

From a study of vertebrate monsters, however, Morrill correctly comes to the following conclusion: "From the evidence at hand, it seems probable that the primary cause of visceral asymmetry in vertebrates is to be sought for at the completion of cleavage rather than in the period of cleavage itself."

Origin of Spiral Growth.

The explanation of spiral epithelial growth is under investigation. Spiral growth involves two motions: a motion of rotation, circular, and a motion of straight progression, rectilinear. The latter is evidently imparted from growing points of mitosis. Growth would

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tend to pursue a straight line if the factors of resistance due to differential growth did not interfere. An exception to this would be found in the spiral type of cleavage observed by Child in *Arenicola cristata*, and Wilson in *Nereis*. In these forms the rotation of the cells appears to be predetermined in the parent cells, as is proved by the position of the spindles and by the form of division. In the intestine the inner curved surface of the mesenchymal wall constantly deflects the mitotic path in a circular manner. This would impart the rotary element to the compound spiral motion of growth.

The relation of the pharynx to the esophagus and of the rectum to the colon is comparable to that of the wide and narrow components of a funnel. A stream running down the inclined wall of the wide element would be deflected in a rotary manner so that its progression through the narrow integer would be in the manner of a helix. The determination of a left- or right-handed helix would depend upon the initial direction of impelling the stream.

The direction of epithelial growth in a left-handed helix has a definite mechanical basis in the intestine, but as yet is undetermined. The epithelial growth is roughly comparable to the stream exemplified above; *i.e.*, the glass of the funnel to the mesenchyme. The initial impulse of the water pressure is comparable to that of growth. This causes the rectilinear motion of progression. The circular resistance of the glass is analogous to that of the mesenchyme. This causes a deflection of the rectilinear motion in a rotary manner, the two characterizing the helicoidal motion of the intestine.

CONCLUSIONS.

Esophageal Development.

1. The region of most active mitosis per mm. of cross-section in the esophagus is the entodermal epithelial tube. The mitotic figures follow a spiral path in the manner of a left-handed helix from the cephalic to the caudal direction.

2. The region of least active growth per mm. of cross-section in the esophagus is the mesenchyme surrounding the epithelial tube.

3. The helicoidal activity of the epithelial tube causes a vortical reaction in the surrounding mesenchyme. The mesenchymal whirlpool represents a reaction to the spirally growing epithelial tube.

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4. In embryos 9.5 to 14 mm. in length the esophageal epithelial tube grows relatively more rapidly in width than in length. During this period the myoblasts which form the inner, close spiral, muscle coat of the esophagus are becoming rapidly differentiated in the outer condensed margin of the mesenchymal maelstrom.

5. The nuclei, first spherical then oval, and finally rod shaped with rounded ends, are drawn out in the direction of the circumference of the mesenchymal rim which is directed tangentially.

6. The cytoplasm is also drawn out in the direction of the mesenchymal rim of the vortex. The elongated rows of isolated granules appear which subsequently, by confluence, form the myofibrillæ. These cytoplasmic derivatives are elongated in the direction of the circumference of the vortex.

7. Between the epithelial tube and the myoblastic rim at the periphery of the mesenchymal whorl is found the embryonic connective tissue. From this direct observation the conclusion is made that an optimum tensional stress stimulus is necessary to elicit the formation of muscular tissue at the circumference of the mesenchymal vortex. Consequently, the formation of a specific derivative from a pluripotent mesenchymal cell is due to the fortuitous circumstance of position.

8. In embryos from 14 to 24 mm. in length, the esophagus grows relatively more rapidly in length than in width. This elongation is due to two factors; first, the descent of the stomach, and, second, the resistance to diametrical growth presented by the inner close spiral musculature. The epithelial tube, still the dominant zone of mitotic activity, pursues the lines of least resistance, and consequently growth in length takes place. This is due to the shifting of the planes of cell division on account of the compression of the inner, close spiral, muscle coat.

9. The undifferentiated mesenchyme peripherad to the inner, close spiral musculature is elongated and the histogenetic changes in muscular formation are gradually taking place between 14 and 24 mm. A very attenuated, outer, elongated, spiral, or longitudinal muscle coat is detected in the esophagus of a 24 mm. pig embryo.

10. The characteristic intestino-colic flexure is a torsional reaction of the mesenchyme. The mesenchymal cells are thrown into a left-handed helicoidal series, corresponding to the activity in the epithelial

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tube. The right-handed helicoidal reaction of the mesenchyme, therefore, is due to the left-handed helicoidal growth of the epithelial tube.

11. The normal asymmetry of the abdominal viscera as well as the position of the gut is dependent upon the clockwise reaction of the stretched mesenchymal cell. These cells are stretched by the left-handed helicoidal growth of the epithelial tube. One factor producing *situs inversus viscerum* could be the reversal of the spiral growth of the epithelial tube resulting in a reaction of the mesenchyme in a direction opposite, namely counterclockwise, to that which occurs normally.

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ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. I.

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I. INTRODUCTION.

It had been shown in a preceding paper[1] that the relative influence of ions on the rate of diffusion of water through collodion membranes can be expressed by arranging the ions in two definite series, as follows:

(1) Rb < K < Na < Li < divalent < trivalent cations.

(2) Cl < Br < I < divalent < trivalent anions.

These results are intelligible on the assumption that we are dealing in this case with electrostatic effects of ions, the electrostatic effects of the cations being due to the excess charges of the positive nucleus and the electrostatic effects of the anions being due to the charges of the captured valency electrons. In this case the relative efficiency of monatomic and monovalent cations should increase inversely with the radius of the ion; and the relative efficiency of the monovalent, monatomic anions should increase directly with the radius of the ion. In the cation series the Li ion should be next to the divalent cations, while in the anion series the iodion should be next to the divalent anions.

This order differs from that usually given for the action of ions on the physical properties of proteins. Hofmeister[2] and afterwards Pauli[3] determined the relative order of efficiency of ions for the precipitation of proteins. This order is according to Pauli:

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 673.

[2] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1888, xxiv, 247.

[3] Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 225; *Fortschr. naturwiss. Forschung*, 1912, iv, 237.

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For cations, Mg < NH_4 < K < Na < Li.

For anions, CNS < I < Br < Cl < acetate < tartrate < citrate < phosphate < sulfate < Fl.

The reader will notice that in the cation series the divalent cation Mg is next to NH_4 and K, while we should expect it to follow Li in order of efficiency. On the other hand, if the efficiency of the monovalent, monatomic cations increases in the order of K < Na < Li as Hofmeister and Pauli observed we should expect the efficiency of the anions to increase in the order of Cl < Br < I instead of in the reverse order. We must conclude that the phenomena of precipitation of proteins by ions are either not determined by the electrostatic forces of the ion or that other variables enter which are not yet known. This latter possibility exists since the concentration of electrolytes required for precipitation is very high and the writer has shown that the influence of ions causing the anomalous diffusion of water through membranes already ceases to be noticeable in comparatively low concentrations of electrolytes.[4]

It therefore seems advisable to restrict our attention to such influences of ions where they act in low concentrations. This is possible when we compare the effects of low concentrations of different acids and alkalies on the physical properties of proteins. The first one who undertook such a study on a larger scale was Pauli. He recognized that in order to investigate the effect of acids and alkalies on proteins it was necessary to free them first from salts. When Pauli and Handovski[5] added to blood albumin dialyzed for 6 weeks various quantities of different acids they found that all the acids increased the viscosity of the blood albumin but in a different degree. Their results are expressed in curves from which we gather that the relative order of efficiency of various acids tried by them was

HCl > monochloracetic > oxalic > dichloracetic > citric > acetic > sulfuric > trichloracetic acid

where HCl raises the viscosity most, and trichloracetic least. The viscosity was measured by the time of outflow through a viscometer. Pauli assumes, as Laqueur and Sackur[6] had done previously in their

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

[5] Pauli, W., and Handovski, H., *Biochem. Z.*, 1909, xviii, 340.

[6] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

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experiments on casein solutions, that the increase in the viscosity of the protein caused by the addition of acid (or of alkali) is due to an increase in the ionization of proteins, as a consequence of the salt formation between protein and acid or alkali, the protein salt being capable of a greater degree of ionization than the protein not treated with acid or alkali. In order to explain the relative differences in the effect of various acids on the viscosity, Pauli assumes that the protein salts formed with different acids differ in their degree of electrolytic dissociation. He states; *e.g.*, that "the strong trichloracetic acid and the slightly weaker sulfuric acid furnish few protein ions."[7] He also states that the relative efficiency of different anions on the other properties of proteins, as osmotic pressure, precipitation by alcohol, is the same as that found for viscosity.

In his experiments on the influence of acids and alkalies on the osmotic pressure of gelatin solutions the writer had arrived at the conclusion that the results he obtained cannot be expressed in terms of ion series.[8] These experiments showed only an influence of the valency of the anion or cation in combination with the gelatin, but no other influence. Solutions of Li, Na, K, and NH_4 gelatinate of the same pH and the same concentration of originally isoelectric gelatin had the same osmotic pressure. The same was true for Ca and Ba gelatinate; but the osmotic pressure of Ba and Ca gelatinate was only one-half or less than that of Li, Na, K, or NH_4 gelatinate of the same pH and the same concentration of originally isoelectric gelatin. The influence of acids on the physical properties of proteins was still more interesting. Solutions of gelatin chloride, bromide, nitrate, acetate, phosphate, citrate, tartrate, succinate, of the same pH and the same concentration of originally isoelectric gelatin, had approximately the same osmotic pressure, while the osmotic pressure of solutions of gelatin sulfate was only half or less than half of that of gelatin chloride, etc. The osmotic pressure of solutions of gelatin oxalate was almost but as a rule not quite as high as that of gelatin chloride.[8]

This peculiarity found its explanation in an investigation of the combining ratios of these acids with gelatin. The writer was able to

[7] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 243.
[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 559.

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show that the strong dibasic acid H_2SO_4 combines in equivalent proportions with gelatin while the weaker acids, *e.g.* phosphoric or oxalic, combine in molecular proportions. At a given pH, *e.g.* pH = 3.5, 1 gm. of originally isoelectric gelatin is in combination with three times as much 0.1 N acid when the acid is H_3PO_4 than when it is HCl; and with almost twice as much 0.1 N acid when the acid is oxalic than when it is HCl; while the ratio of HCl and H_2SO_4 is 1. It follows from this that the strong dibasic acid H_2SO_4 forms a salt with gelatin in which the anion is divalent, namely SO_4, while the weak tribasic acid H_3PO_4 forms a salt with gelatin in which the anion is monovalent; namely, H_2PO_4 (instead of the trivalent anion PO_4). Likewise, citric, succinic, and tartaric acids form gelatin salts in which the anion is always monovalent; namely, H-succinate, H-tartrate, etc. In the case of oxalic acid this is also the case though a slight amount of gelatin salt with divalent anion is probably formed and this might account for the fact that the osmotic pressure of gelatin oxalate is generally slightly less than that of gelatin chloride.[9]

It was also found that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent and not in molecular proportion thus showing that the cation of Ca gelatinate and Ba gelatinate is divalent.[8]

All these facts together show that solutions of salts of gelatin with a bivalent ion have an osmotic pressure of one-half or less of that of solutions of salts of gelatin with monovalent ions at the same pH and the same concentration of originally isoelectric gelatin.

This then proves that while the valency of the ion in combination with the gelatin has a strong influence on the osmotic pressure of the gelatin solution differences in the nature of ions of the same valency have either no effect on the osmotic pressure or if they have such an effect it is too slight to be noticeable by our method of experimentation. We can certainly say that it is impossible to express our observations in terms of the Hofmeister series. In the Hofmeister series phosphates and chlorides stand at almost opposite ends of the anion series while in our experiments the effects of H_2PO_4 and HCl are identical if we compare the effects at the same pH of the gelatin solutions. The reason that Pauli arrived at a different conclusion lies

[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483.

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probably in the fact that he compared the effects of the addition of equal quantities of acid to a protein while we compared the effects of different acids at equal hydrogen ion concentrations of the solutions.

II. Action of Weak and Strong Monobasic Acids.

We will first indicate why it is necessary to choose equal hydrogen ion concentrations as a standard of comparison instead of using equal quantities of acid. We always use as standard material isoelectric protein, to which we add enough acid or alkali to bring it to the desired hydrogen ion concentration. In the case of gelatin we proceed usually in this way; we add to 1 gm. of powdered gelatin brought to the isoelectric point (pH = 4.7) small quantities of acid to bring the samples to the desired hydrogen ion concentration, then melt the gelatin, and bring the volume of the solution to 100 cc. by adding H_2O. The pH is then determined. It is hardly necessary to state that it requires greater quantities of weak than of strong acid to bring the gelatin to the same pH on the acid side of the isoelectric point. Fig. 1 gives the quantities of 0.1 N acetic, monochloracetic, dichloracetic, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin in a 1 per cent solution to the same pH. The abscissæ in Fig. 1 are the pH of the gelatin solution resulting from the addition of acid and the ordinates are the numbers of cc. of 0.1 N acid which must be contained in 100 cc. of a 1 per cent solution of originally isoelectric gelatin to produce the pH. The curve indicates that the quantity of acid required is the less the stronger the acid. If we now measure the osmotic pressure of the solutions of the four gelatin acetates (all 1 per cent in regard to the originally isoelectric gelatin) and if we plot the curves with the values for the osmotic pressure as ordinates and the pH as abscissæ, the curves for all four gelatin-acetate salts are practically identical (Fig. 2). All four curves have a minimum at the isoelectric point pH = 4.7; they all rise identically with a diminution in pH (*i.e.* a rise in hydrogen ion concentration); all reach their maximum at a pH of about 3.5, and all drop almost as steeply with a further diminution of pH as they rise on the ascending side of the curve. The drop will not be discussed in this paper.

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The slight differences in the maximal height of the four curves are within the limits of the accuracy of these experiments, and the same or even greater variations may be observed when the same experiment is repeated several times with the same acid. The maximal values of osmotic pressure reached in these experiments at pH 3.5 are the same as those reached with gelatin salts of any other monovalent anion, Cl, Br, NO_3, phosphate, citrate, succinate, etc.

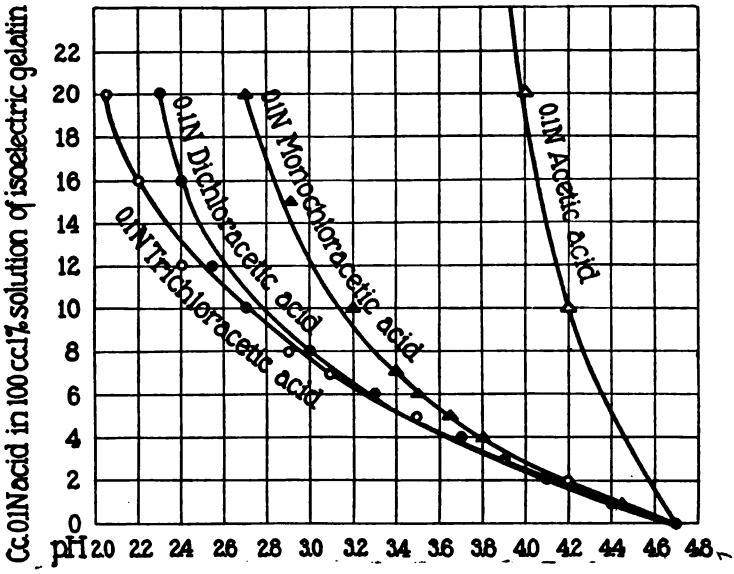

FIG. 1. The ordinates represent the number of cc. of 0.1 N acetic, mono-, di-, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin to the pH indicated by the abscissæ. Enough H_2O was added to bring the gelatin-acid solution to a volume of 100 cc.

It follows from this that the conclusions which are based upon a comparison of the quantities of the acid added instead of upon the pH of the protein solutions cannot be correct.

The reason why we get identical curves when we plot the osmotic pressures as ordinates over the pH as abscissæ seems to be as follows.

When we add a definite small quantity of acid to 1 gm. of isoelectric gelatin, melt, and bring the volume of the solution to 100 cc. by adding H_2O, part of the acid will combine with gelatin and part will remain

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free in the solution. The more acid we add, the greater the amount of isoelectric gelatin transformed into gelatin-acid salt. We assume that there is a definite equilibrium between the hydrogen ion concentration of the solution, the amount of gelatin-acid salt formed, and isoelectric gelatin. If this view is correct, it follows that at the same

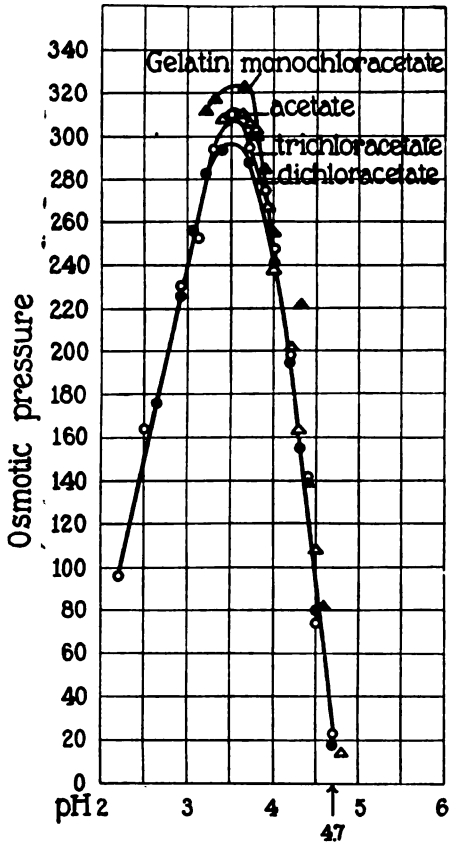

FIG. 2. The ordinates indicate the osmotic pressure (in mm. of the height of a column of the gelatin solution) of 1 per cent solutions of originally isoelectric gelatin which have been brought to different pH by the addition of the acids used in Fig. 1. The abscissæ are the pH. The curves are practically identical, the slight differences at the summit being inside the range of the variation found for the same acids.

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pH and with the same concentration of originally isoelectric gelatin all mixtures of acid and gelatin solution must have the same concentration of gelatin-acid salt; and if this is the case they must all have the same osmotic pressure if the valency of the anion is the same, since the osmotic pressure is almost entirely determined by the gelatin-acid salt, that of the isoelectric gelatin being very low. This conclusion is in harmony with the view expressed by Laqueur and Sackur, Pauli, and others. The writer differs from Pauli only in regard to the statement that the different acids, *e.g.* HCl, H_3PO_4, tartaric, acetic, and trichloracetic, have different effects on the physical properties of proteins.

III. Combining Ratios of Acids and Alkalies with Crystalline Egg Albumin and the Osmotic Pressure of the Albumin Solutions.

Crystalline egg albumin was prepared under Dr. Northrop's supervision according to Sörensen's method,[10] and crystallized three times. The only difference in procedure was in the dialysis. Instead of putting the water under negative pressure as was done by Sörensen, pressure was put on the egg albumin by attaching a long glass tube full of water to the dialyzing bag so that the solution was under about 150 cm. water pressure during dialysis. This was necessary to avoid too great an increase in volume. The same stock solution of albumin served for all the experiments and was diluted before the experiment to a 1 per cent solution. The concentration of ammonium sulfate left in the solution was between M/1,000 and M/2,000. The pH of the stock solution was about 5.20. By adding about 1 cc. of 0.1 N HCl to 100 cc. of a 1 per cent solution of this albumin the solution was brought to the isoelectric point of the egg albumin, which is according to Sörensen at pH = 4.8.

The 1 per cent solutions were made up with different quantities of acid (or alkali) and the pH of the albumin solution was determined electrometrically.[11] In Fig. 3 are plotted the curves in which the pH are the abscissæ and the cc. of 0.1 N acid required to obtain the various

[10] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 1.

[11] The colorimetric determination which gives fairly good results in the case of gelatin is unreliable in the case of egg albumin.

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pH as ordinates. The curves represent these values for four acids, HCl, H_2SO_4, H_3PO_4, and oxalic acid. Beginning with the lowest curve we notice that the curve is the same for 0.1 N HCl and 0.1 N H_2SO_4, since both are strong acids; or, in other words, H_2SO_4 combines

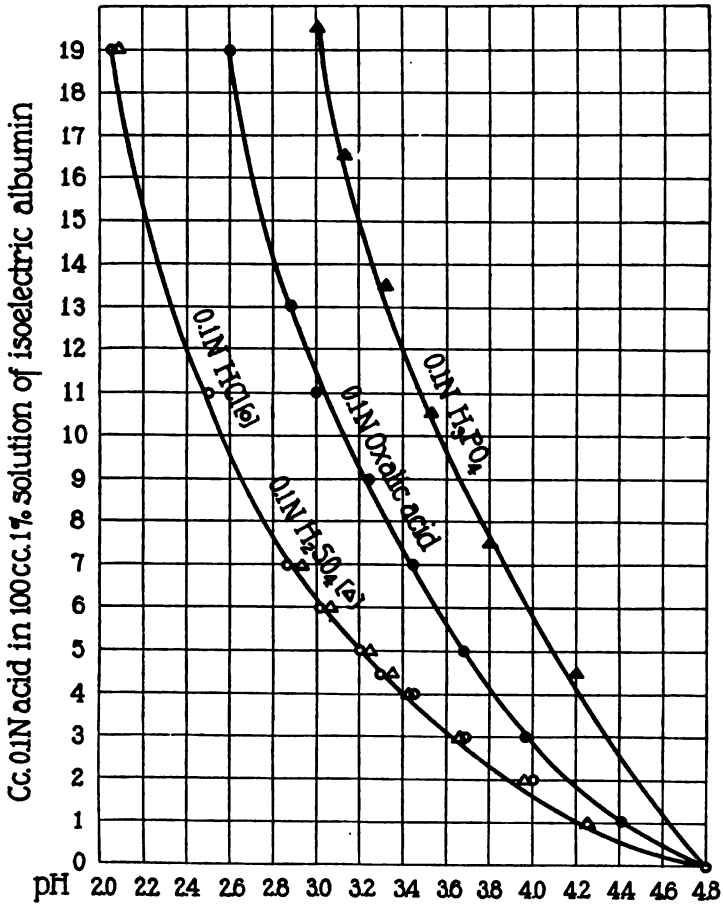

FIG. 3. The ordinates represent the number of cc. of 0.1 N HCl, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissæ. Enough H_2O was added to bring the solutions of albumin and acid to a volume of 100 cc. For the same pH the ordinates for HCl, H_2SO_4, and phosphoric acid are approximately as 1 : 1 : 3. The ratio of HCl : oxalic acid is a little less than 1 : 2.

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in equivalent proportions with egg albumin. The curve for H_3PO_4 is the highest curve and if we compare the values for H_3PO_4 with those for HCl (or H_2SO_4) we notice that for each pH the ordinate for H_3PO_4 is as nearly three times as high as that for HCl as the accuracy of our experiments permits. This means that under the conditions of our experiments phosphoric acid combines with albumin in molecular proportions and that the anion of albumin phosphate is the monovalent anion H_2PO_4.

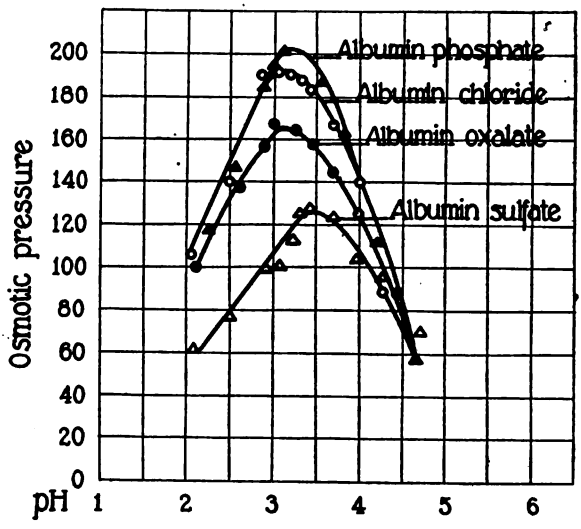

FIG. 4. Osmotic pressure of different albumin-acid salts. The ordinates indicate the osmotic pressure (in mm. of 1 per cent albumin solution); the abscissæ are the pH. All solutions are 1 per cent in regard to isoelectric albumin. The curves for albumin chloride and albumin phosphate are identical.

The values for oxalic acid are for pH above 3.2 not quite twice as high as those for HCl, indicating that for these values of pH oxalic acid combines to a greater extent in molecular and only to a smaller extent in equivalent proportions with albumin.

If egg albumin behaves like gelatin we should expect that the curves of osmotic pressure for albumin phosphate and albumin chloride when plotted as a function of the pH should be identical, since the anion is in both cases monovalent; that the curve for albumin sulfate should

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be considerably lower since the anion SO_4 in combination with albumin is bivalent; while the curve for the osmotic pressure of albumin oxalate should be between the curves for albumin chloride and albumin sulfate, but much nearer the chloride than the sulfate curve. Fig.

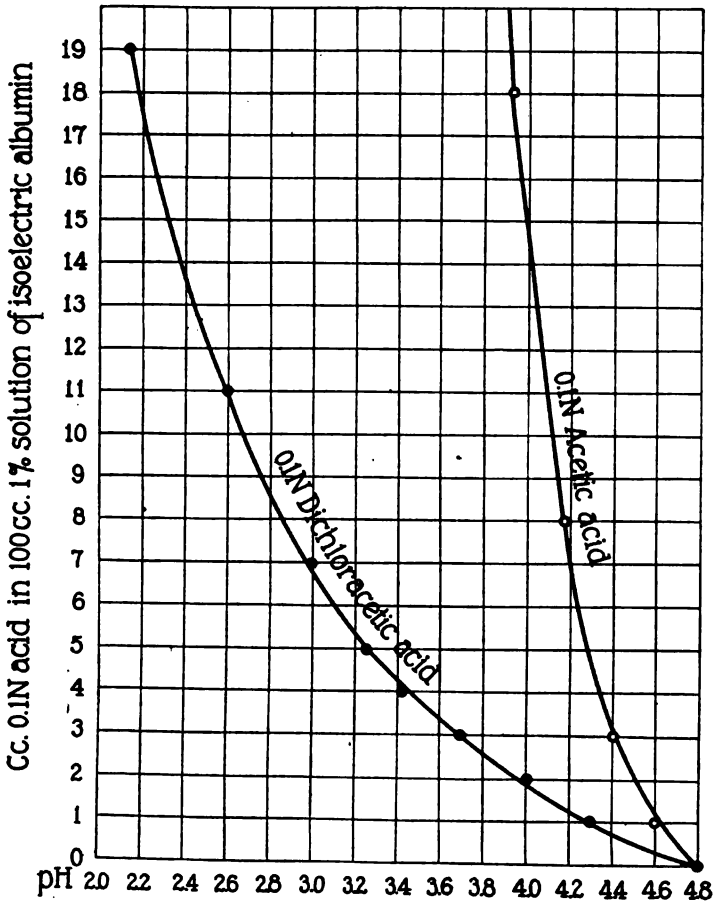

FIG. 5. Curves expressing the cc. of 0.1 N acetic and dichloracetic acids required to bring 1 gm. of isoelectric albumin to different pH.

4 giving the curves of the osmotic pressures of the four albumin salts shows that this expectation is fulfilled.

Fig. 5 gives the curves for the combining ratios of acetic acid and dichloracetic acid with isoelectric albumin, showing the same differ-

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ence as the corresponding curves for gelatin in Fig. 1. Fig. 6 gives the curves for the influence of the two acids upon the osmotic pressure of 1 per cent solutions of originally isoelectric albumin. The two curves are identical and are also identical with those of albumin chloride and albumin phosphate in Fig. 4, thus confirming our theory.

In Fig. 7 are given the curves for combining ratios of NaOH, $Ca(OH)_2$, and NH_4OH with isoelectric albumin. The curve for $Ca(OH)_2$ is identical with that for the strong base NaOH, indicating that $Ca(OH)_2$ combines with egg albumin in equivalent proportions.

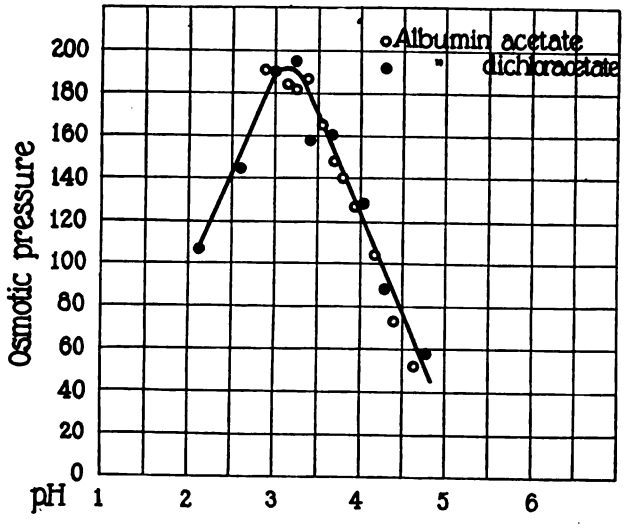

FIG. 6. Curves of osmotic pressure of solutions of albumin acetate and albumin dichloracetate. The curves for both acids are identical.

Fig. 8 shows that the curve for the osmotic pressure of Ca albuminate is only one-half as high as that of Na albuminate as was to be expected. The curve for NH_4 albuminate is identical with that for Na albuminate, which was to be expected since the NH_4 is monovalent.

The results with albumin are therefore identical with those obtained in the case of the corresponding gelatin salts. The result that gelatin phosphate and albumin phosphate behave like gelatin chloride and albumin chloride may be considered as a crucial test against the colloidal conception of ion effects on proteins and in favor of a purely chemical theory.

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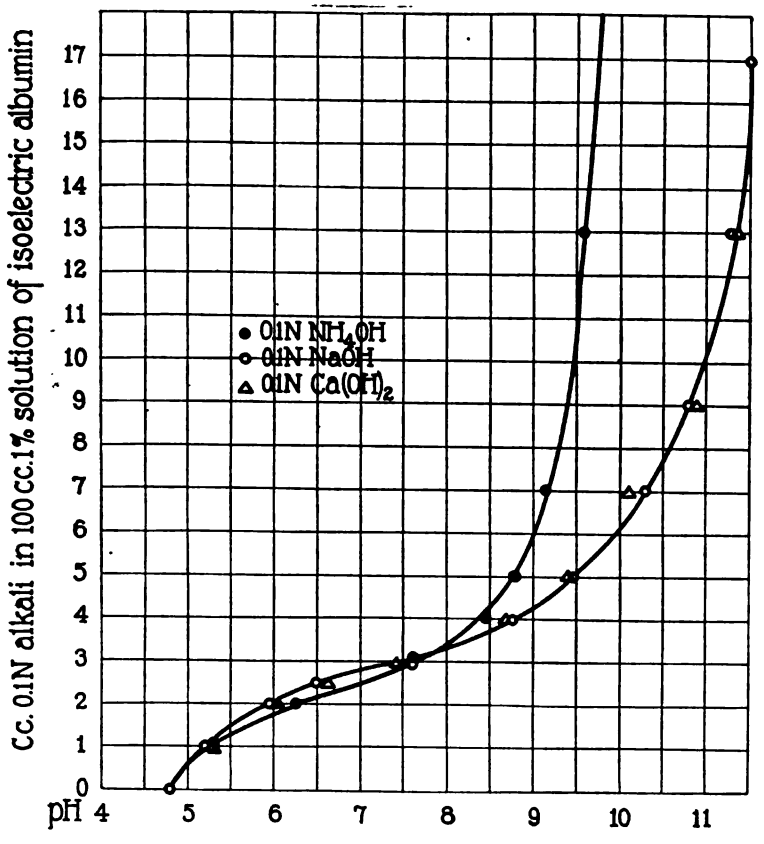

FIG. 7. Curves representing the number of cc. of 0.1 N NH_4OH, NaOH, and $Ca(OH)_2$ required to bring 1 gm. of isoelectric, crystalline egg albumin to different pH. The curves for NaOH and $Ca(OH)_2$ are identical.

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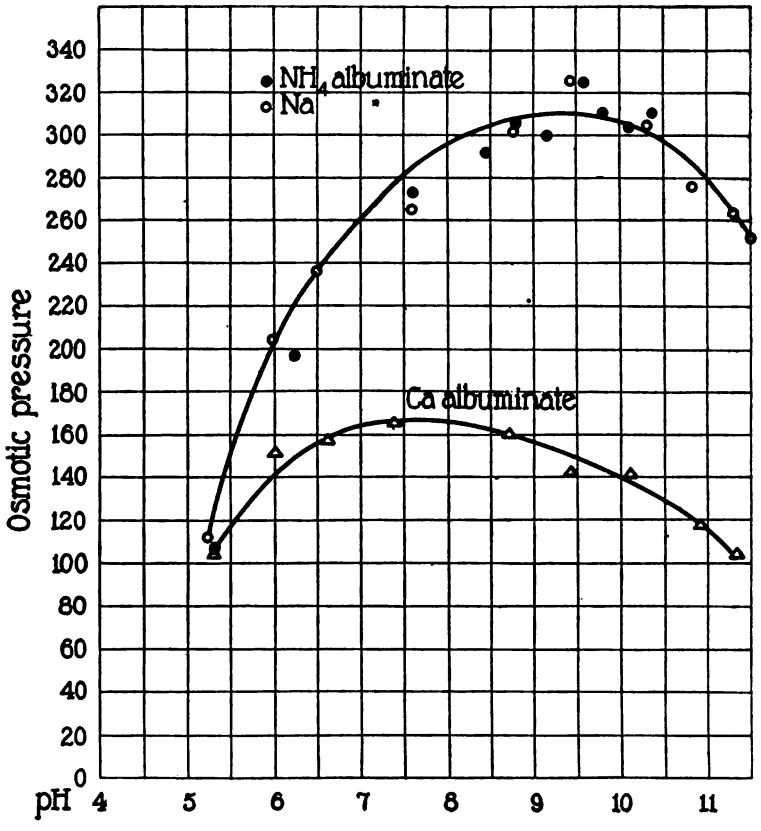

FIG. 8. Curves of the osmotic pressure of NH_4, Na, and Ca albuminate at different pH. The curves for NH_4 and Na albuminate are practically identical.

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IV. Combining Ratios of Acids and Bases with Gelatin and the Viscosity of Gelatin Salts.

Since Pauli's ion series was based primarily on the influence of acids or their anions on the viscosity of protein solutions it seemed necessary to find out whether or not viscosity measurements confirm the conclusions at which we arrived on the basis of osmotic pressure experiments on gelatin and egg albumin. A few remarks concerning our method are required.

The gelatin is first rendered isoelectric in the following way. 25 gm. of powdered gelatin of pH about 7.0 are put into 1 liter of M/128 acetic acid for 30 minutes at 10°C., after which time the acetic acid is renewed and left in contact with gelatin again for 30 minutes at 10°C. The acid is then decanted and replaced with distilled water of about 5°C. The mixture is filtered in a Buchner funnel through muslin, employing slight suction. The gelatin is then washed about six times with 100 cc. of distilled water of 5° each, and is made into a 5 per cent solution which serves as a stock solution. The pH of this solution is about 4.7, or, in other words, the gelatin is isoelectric.

Some of the stock solution is heated to 45° and made up to a 2 per cent solution in quantity sufficient for a day's experiments. This 2 per cent solution is kept during the day at 24°C. To 50 cc. of this solution is added the desired acid or alkali in sufficient quantity and then the volume is raised to 100 cc. by the addition of enough distilled water. This 1 per cent solution is then rapidly brought to a temperature of 45°, kept there for 1 minute, and is then rapidly cooled to 24°. The solution is stirred constantly during the heating and cooling. The viscosity is measured immediately after the solution is cooled to 24°C. The measurements were all made at 24°C. by using the time of outflow through a viscometer. The time of outflow of distilled water through an Ostwald viscometer at 24°C. was exactly 1 minute. Each measurement of viscosity was repeated with the same gelatin solution and the beginning and the end of a series consisted in the measurement of viscosity of isoelectric gelatin. These latter measurements agreed in all experiments within 1 second varying only between 80 and 81 seconds, thus guaranteeing the reproducible character of the experiments.

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The results can be given briefly. Fig. 9 gives the curves for the cc. of 0.1 N HNO_3, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH. The curve shows again that for each pH the number of cc. of 0.1 N acid required are for HNO_3, H_2SO_4, oxalic, and phosphoric acids approximately as 1:1:2:3.

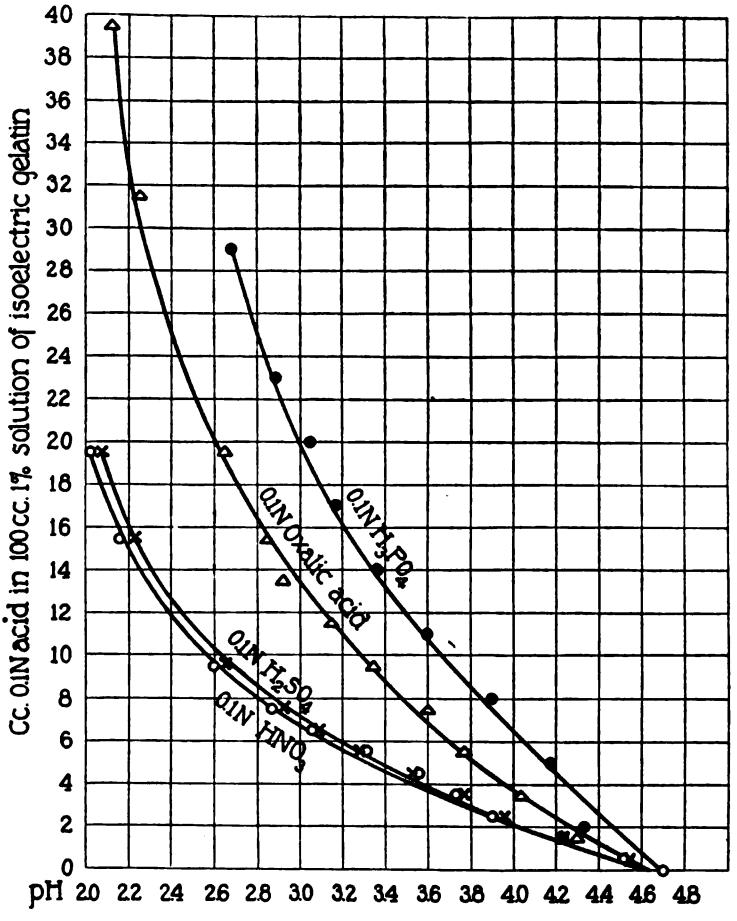

FIG. 9. Curves for the number of cc. of 0.1 N HNO_3, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). Curves similar to those for egg albumin (Fig. 3). For the same pH the ratio of HNO_3, H_2SO_4, oxalic, and phosphoric acids required is approximately as 1:1:2:3.

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Fig. 10 gives the curves for the viscosity of 1 per cent solutions of gelatin chloride, sulfate, oxalate, and phosphate. The abscissæ are the pH, the ordinates the ratio of the time of outflow of the gelatin solutions divided by the time of outflow of pure water. For the sake

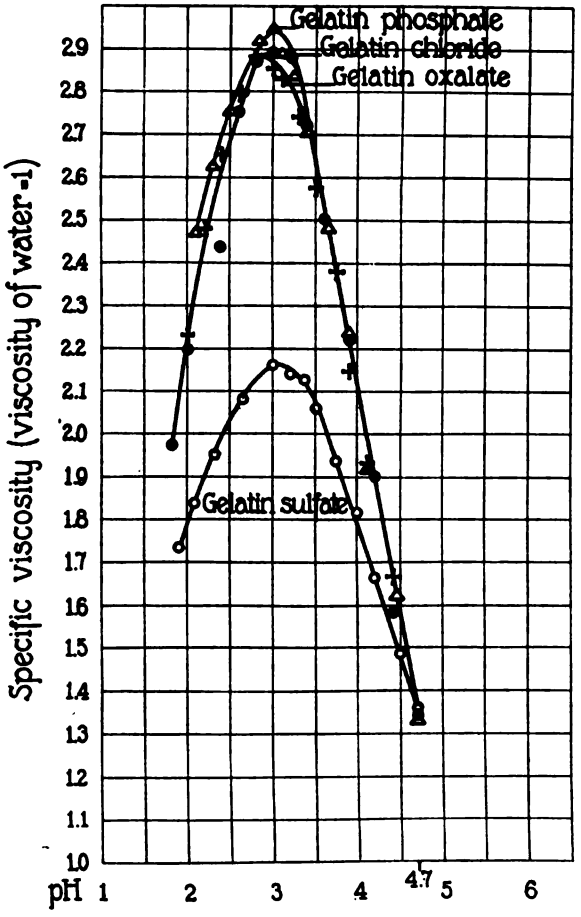

FIG. 10. The curves of specific viscosity of 1 per cent solution of originally isoelectric gelatin brought to different pH by the acids mentioned in legend of Fig. 9 except that HCl is used for HNO_3. The curves of viscosity for gelatin chloride, phosphate, and oxalate are practically identical. Specific viscosity given as time of outflow of gelatin solution divided by time of outflow of water through viscometer at 24°C.

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of brevity we will call this quotient the specific viscosity of the gelatin solution. The curves for the four acids all rise steeply from the isoelectric point with increasing hydrogen ion concentration until they reach a maximum at pH about 3.0. The curves then drop again.

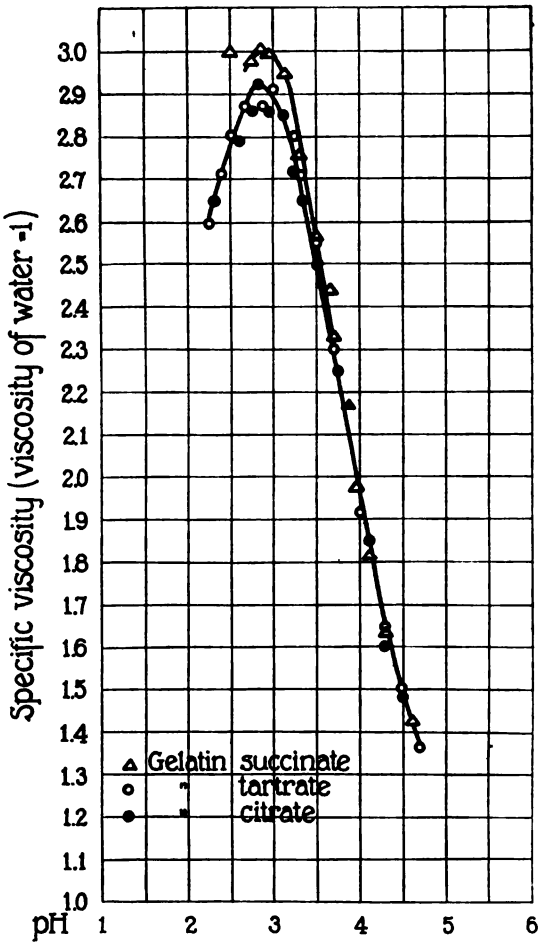

FIG. 11. Curves of specific viscosity of gelatin succinate, tartrate, and citrate. The curves are practically identical with those for the viscosity of gelatin chloride and phosphate.

The curves for the three acids, gelatin chloride, oxalate, and phosphate, are practically identical while the curve for gelatin sulfate is considerably lower.

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Fig. 11 gives the curves for the viscosity of gelatin citrate, tartrate, and succinate. The three curves are practically identical and also identical with the curves for gelatin chloride and gelatin phosphate in Fig. 10.

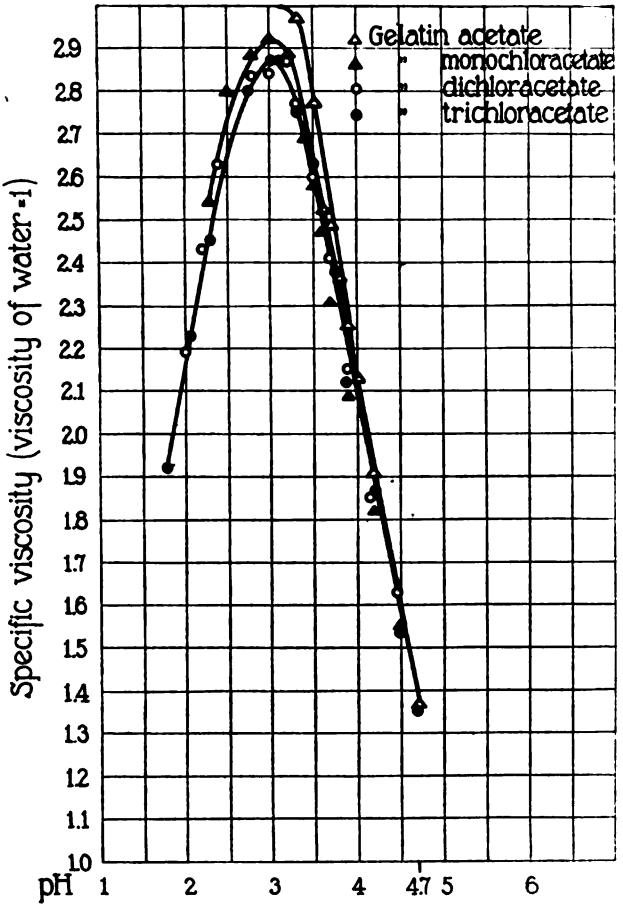

FIG. 12. Curves of specific viscosity of gelatin acetate, mono-, di-, and trichloracetate. Curves identical with those for gelatin chloride and phosphate.

Fig. 12 gives the curves for the viscosity of 1 per cent solutions of originally isoelectric gelatin to which acetic and mono-, di-, and trichloracetic acids have been added. The curves are again identical

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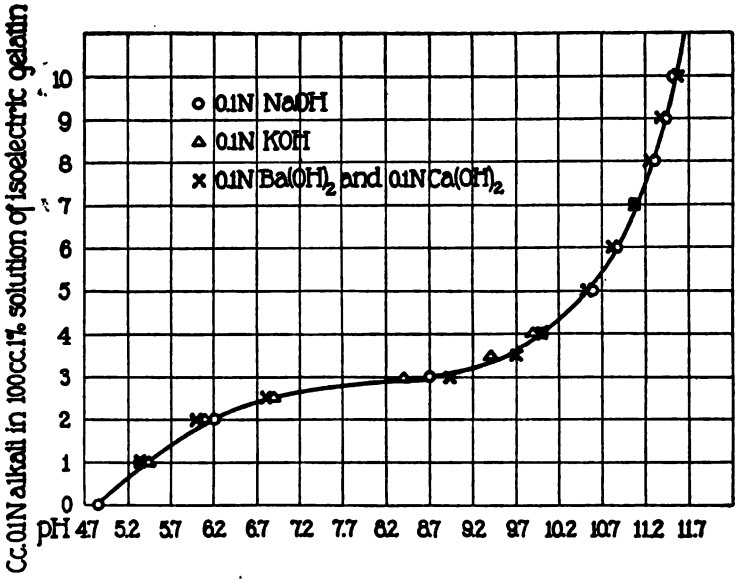

FIG. 13. Curves for the number of cc. of 0.1 N NaOH, KOH, $Ba(OH)_2$, and $Ca(OH)_2$ required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). All four curves are identical.

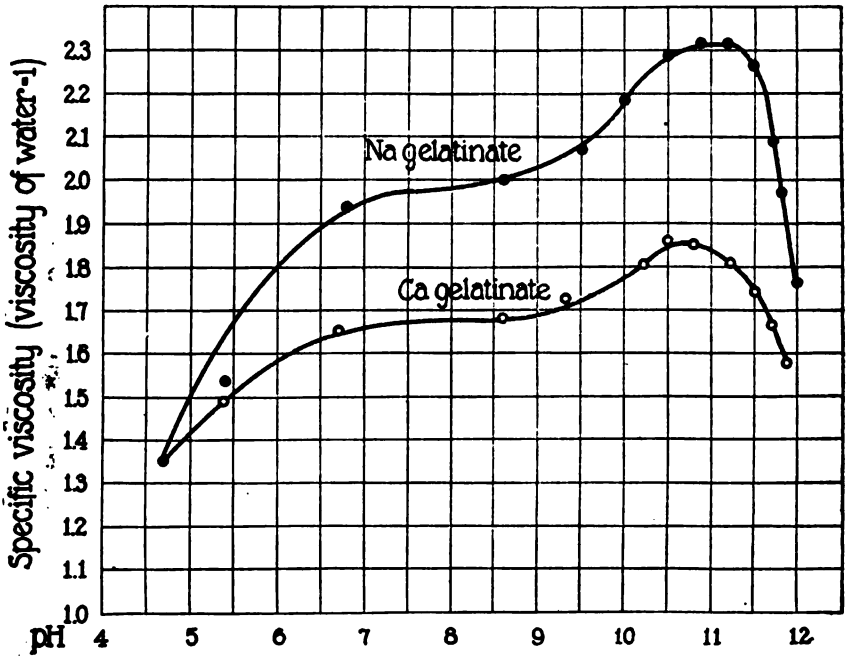

FIG. 14. Curves for specific viscosity of Na and Ca gelatinate for different pH.

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with those for gelatin chloride, phosphate, etc. These curves are interesting in connection with Pauli's statement that trichloracetic acid causes the same low values of viscosity as sulfuric acid. This was the case neither in our experiments on the osmotic pressure nor in those on the viscosity of gelatin solutions.

Fig. 13 gives the cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ required to bring 1 gm. of originally isoelectric gelatin in 100 cc. solution to a given pH. One curve suffices for the four alkalies thus proving that Ca and Ba combine with gelatin as bivalent ions. We should expect the curve for the viscosity of Ca gelatinate to be considerably lower than that of Na gelatinate. Fig. 14 shows that this is true.

SUMMARY.

1. This paper contains experiments on the influence of acids and alkalies on the osmotic pressure of solutions of crystalline egg albumin and of gelatin, and on the viscosity of solutions of gelatin.

2. It was found in all cases that there is no difference in the effects of HCl, HBr, HNO_3, acetic, mono-, di-, and trichloracetic, succinic, tartaric, citric, and phosphoric acids upon these physical properties when the solutions of the protein with these different acids have the same pH and the same concentration of originally isoelectric protein.

3. It was possible to show that in all the protein-acid salts named the anion in combination with the protein is monovalent.

4. The strong dibasic acid H_2SO_4 forms protein-acid salts with a divalent anion SO_4 and the solutions of protein sulfate have an osmotic pressure and a viscosity of only half or less than that of a protein chloride solution of the same pH and the same concentration of originally isoelectric protein. Oxalic acid behaves essentially like a weak dibasic acid though it seems that a small part of the acid combines with the protein in the form of divalent anions.

5. It was found that the osmotic pressure and viscosity of solutions of Li, Na, K, and NH_4 salts of a protein are the same at the same pH and the same concentration of originally isoelectric protein.

6. $Ca(OH)_2$ and $Ba(OH)_2$ form salts with proteins in which the cation is divalent and the osmotic pressure and viscosity of solutions of these two metal proteinates are only one-half or less than half of

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that of Na proteinate of the same pH and the same concentration of originally isoelectric gelatin.

7. These results exclude the possibility of expressing the effect of different acids and alkalies on the osmotic pressure of solutions of gelatin and egg albumin and on the viscosity of solutions of gelatin in the form of ion series. The different results of former workers were probably chiefly due to the fact that the effects of acids and alkalies on these proteins were compared for the same quantity of acid and alkali instead of for the same pH.

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THE RECOVERY OF TRANSMISSIVITY IN PASSIVE IRON WIRES AS A MODEL OF RECOVERY PROCESSES IN IRRITABLE LIVING SYSTEMS.

PART I.

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(Received for publication, June 20, 1920.)

I. INTRODUCTORY.

A striking feature in the phenomena of activation in passive iron wires immersed in solutions of nitric acid is that in acid above a certain critical concentration the reaction is a *temporary* one, followed immediately by an automatic return of the metal to the passive state.[1] Activation initiated at any region of such a wire is thus transmitted rapidly along its whole length in a wave-like manner, each region as it becomes active activating the region next adjoining (by means of the local electric circuit between the passive and active areas) and immediately becoming itself again passive. In this tendency to revert promptly to the chemically inactive or passive state after activation the passive metal resembles an irritable and conducting living element such as a nerve fiber or muscle cell. In order to maintain activity in the living system, constant repetition of stimulation is necessary; and similarly chemical activity in a passive wire immersed in a sufficiently strong solution of nitric acid (55 or more volumes per cent of HNO_3, specific gravity 1.42) is an automatically self-limiting process which can be maintained only by repeated contact of the activating metal; *e.g.*, zinc. Even under such conditions only a partial and irregular activity is possible, which is confined to the immediate neighborhood of the contact; *i.e.*, it fails to be transmitted through more than a short distance. This behavior is highly

[1] For a fuller description of this phenomenon *cf.* Lillie, R. S., *Science*, 1918, xlviii. 51.

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characteristic; after a wire has been activated in the usual manner and has reverted to the passive state it is found impossible to reactivate it as a whole by a single contact until a certain interval has elapsed, the duration of which varies with the concentration of acid and with the temperature in a manner to be described later. At first, touching with zinc causes only a brief local reaction which is confined to an area of 1 or 2 cm. from the contact; a second trial made somewhat later gives a reaction which spreads more rapidly and through a greater distance; and with further successive trials the distance through which the activation wave travels, as well as its speed, increases by degrees until eventually rapid transmission through an indefinite distance becomes again possible.

This failure of complete activation and transmission for a certain period succeeding the passage of an activation wave may be compared with the similarly inexcitable and non-conductive interval or "refractory period" of irritable living systems. It is well known that a decline or disappearance of irritability and conductivity during a certain interval succeeding the response to stimulation is a constant feature of the excitation process in all irritable tissues.[2] The duration of this interval varies with the nature of the tissue; the most notable correlation is that it is brief in tissues with rapid rate of response (*i.e.* brief chronaxie), such as voluntary muscle and nerve, and relatively prolonged in slowly responding tissues like the heart or involuntary muscle. In certain cases, as in the photoreceptors of mollusks, the period of diminished sensitivity following the response may last for several minutes;[3] such a phenomenon suggests a fatigue effect, and there are various significant resemblances between the refractory period and cases of brief or evanescent fatigue; thus the period is lengthened by conditions which delay recovery from fatigue, such as lack of oxygen,[4] and also by repeated stimulation, as shown in the

[2] The relation of the refractory period to the excitation process in general is well discussed in the Croonian lecture of Lucas, K., *Proc. Roy. Soc. London, Series B*, 1912, lxxxv, 495.

[3] For a description of this phenomenon *cf.* Hecht, S., *J. Gen. Physiol.*, 1918–19, i, 545.

[4] Verworn, M., Allgemeine Physiologie, Jena, 4th edition, 1903, 559. Fröhlich, F. W., *Z. Physiol.*, 1904, iii, 468.

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prolonged "compensatory pause" following an "extra-contraction" in heart muscle.

It is generally assumed that the refractory phase corresponds essentially to the period required for recovery or recuperation. The act of stimulation appears to involve the more or less complete breakdown or removal of some material or structure which is essential to stimulation; the refractory interval represents a period of reconstruction or restoration. When this process of repair is rapid the refractory period is brief, and *vice versa*. In any single tissue the rate of this reconstruction exhibits a general parallelism with the rate of the structural or chemical breakdown associated with stimulation; hence rapidly responding tissues have as a rule brief refractory periods. This parallelism can, however, be artificially disturbed, as in Tait's experiments, by drugs like protoveratrine and yohimbine which abnormally prolong the refractory period. In such cases an abnormal prolongation of the descending or return phase of the bioelectric variation is also observed.[5] There appears thus to be a correlation between the length of the refractory period and the duration of the bioelectric variation, especially of the return phase of the latter. The relation, however, is not simple, for it is certain that in some cases, *e.g.* heart muscle, the whole period of diminished excitability (including both the "absolute" and the "relative" refractory phases) may greatly outlast the electrical variation.[6] This is also true of the metallic model under consideration in the present paper. It is thus not sufficient to regard the entire refractory phase as corresponding to the time required to restore the normal or "resting" semipermeability and polarizability of the altered plasma membranes of the irritable elements. Recovery of semipermeability is no doubt necessary to a recovery of irritability, since electrical stimulation requires polarizability in the membranes;[7] but some further change appears also to be essential. This question will be discussed later, after the processes in the metallic model

[5] Tait, J., *Quart. J. Exp. Physiol.*, 1910, iii, 221.

[6] Trendelenburg, W., *Arch. ges. Physiol.*, 1912, cxliv, 39. The case of nerve, with a brief refractory period, is similar (*cf.* Adrian, E. D., *J. Physiol.*, 1914, xlviii, 453).

[7] The inference from the work of Nernst, Lapicque, Lucas, Hill, and others on electrical stimulation.

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have been considered. It seems probable that the presence of a brief "absolute," preceding a longer "relative," refractory phase is an index of the existence of two distinct stages or processes in the reconstitution of the plasma membrane, the first stage preceding and the second succeeding the recovery of semipermeability. This view, however, accords imperfectly with Tait's special hypothesis that the "absolute" refractory period corresponds to the period of upstroke of the bioelectric variation, and the "relative" period to the return phase. In the metallic model the change of potential accompanying repassivation, *i.e.* the return phase of the whole electrical variation associated with the local reaction, is a definite index of the reformation of the passivating surface film of oxygen compound. This process occurs rapidly; but it is not until the lapse of a considerable time (usually some minutes) after its completion that the condition of the film becomes such as to permit again of ready and complete transmission. Evidently some further change in the newly restored film is necessary for a return of its original properties; and it seems probable that in the living system closely comparable conditions may obtain. It is certain that the relative refractory period greatly outlasts the return phase of the bioelectric variation in many cases.

In the living irritable element there is evidence that stimulation is associated with a temporary alteration or breakdown of the protoplasmic surface film or plasma membrane, involving a general increase of permeability.[8] Stimulation follows any sufficiently rapid and extensive change in the electrical polarization of the semipermeable protoplasmic surface, or any sufficient local mechanical or chemical alteration; such a change, even though purely physical in itself, initiates a chemical disturbance, associated with a variation of potential, which sweeps over the cell surface, and apparently consists essentially in a wave of alternate dissolution and reconstruction of the surface film. This "propagated disturbance"[9] is the condition for the release of the chemical and other activities in the cell interior which constitute

[8] For a brief summary of this evidence *cf.* Lillie, R. S., *Am. J. Physiol.*, 1915, xxxvii, 356. Further references there given.

[9] Keith Lucas' term, to distinguish the transmitted effect from the local or initiatory effect produced by the stimulating agent. For the experimental basis of this distinction *cf.* Adrian, E. D., and Lucas, K., *J. Physiol.*, 1912, xliv, 68.

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the response to stimulation. Normally in a highly irritable element, such as a muscle cell or nerve fiber, the whole cell surface is thus involved, hence the "all or none" character of the response; but the passage of the excitation wave, and with it the local response, can be interfered with or prevented by various artificial conditions (narcosis, electrotonus, local chemical or mechanical conditions). There are many purely physiological indications that the transmission of excitation from one region of the conducting element to the next adjoining is due to the local bioelectric variation resulting from this surface alteration.[8] The reference of the bioelectric variation to a local change in the physical and chemical character of the cell surface, which apparently acts like an electrode of variable chemical composition and potential, is consistent with what we know of the conditions in various inorganic systems exhibiting an analogous type of behavior; examples of such systems are mercury in hydrogen peroxide,[10] chromium and other electrodes exhibiting rhythmical changes of potential,[11] and especially metals in the passive state, more particularly iron.[12]

Activation and transmission in passive iron are due to alterations in the continuous or impermeable[13] surface film of oxidation product covering the surface of the metal. On account of the extreme thinness of this film, the surface of the passive metal, in its property as an electrode, is highly variable and sensitive to mechanical or other disturbance. The susceptibility of a passive iron wire to activation by mechanical and chemical agents and by the electric current (when the metal is made cathode) is thus readily explained. Interruption of the film produces a local circuit, and electrolyses at the electrode areas of this circuit—reduction at the cathodal and reoxidation at the anodal areas—are responsible both for the rapid extension of the active area (transmission) and for the automatic return to the passive

[10] Bredig, G., and Weinmayr, J., *Z. physik. Chem.*, 1903, xlii, 601. Bredig, G., and Wilke, E., *Biochem. Z.*, 1908, xi, 67. Von Antropoff, A., *Z. physik. Chem.*, 1908, lxii, 513.

[11] Ostwald, W., *Z. physik. Chem.*, 1900, xxxv, 33, 204.

[12] The general article by Bennett and Burnham (Bennett, C. W., and Burnham, W. S., *J. phys. Chem.*, 1917, xxi, 107) gives a full account of the phenomena of passivity in metals and an exhaustive literature list.

[13] *I. e.* impermeable to acid, hence its protective influence.

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state. The resemblances between the phenomena of activation and transmission in the passive iron model and in living protoplasm are thus to be referred to the presence, in both systems, of surface films which readily undergo chemical and structural alteration, these alterations producing changes in the electromotor properties of the surface. Such local alterations have further chemical effects, since they produce local electric circuits which furnish the conditions for electrolysis, and these chemical effects are often extensive because of their automatic tendency to spread wherever a continuous and uniform surface film is present.

The general biological significance of these properties of surface films, especially in relation to the film-pervaded or emulsion-like structure of living protoplasm—emulsions being systems whose properties are determined by the presence of surface films of soap or other material—will be partly evident from the foregoing, but will not be considered in detail in the present paper.[14] It is essential to note, however, that recovery of irritability after stimulation depends (on the foregoing theory) upon the restoration of the altered protoplasmic surface film to its previous condition. It seems most likely that an essentially new film is formed after each stimulation; this process of reconstruction probably involves a specific chemical resynthesis, in addition to a purely physical process of redistribution or rearrangement of surface active compounds. The importance of the chemical factor is indicated both by the dependence of recovery on oxygen and by its high temperature coefficient.[15] In the passive iron model the factors in the recovery of transmissivity are simpler than in protoplasm; but repassivation is known to depend upon the formation of a new surface film of oxidation product; and immediately after the redeposition of this film its properties are different from those which it attains later. We can in fact clearly distinguish two distinct processes in the recovery of passive iron; one the redeposition of the film, and the other the progressive alteration of the newly deposited film until the final state of complete transmissivity is attained. The first process

[14] For an account of various biological analogies in the behavior of the surface film of passive iron, *cf.* Lillie, R. S., *Science*, 1919, l, 259, 416.

[15] Bazett, H. C., *J. Physiol.*, 1907–08, xxxvi, 414. Adrian.[6]

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is associated with a change in the electrical potential of the metallic surface, from that characteristic of active iron (in contact with acid) to that of the passive or oxide-covered surface. This variation of potential is a direct and readily observable index of the return to the passive state; it occurs simultaneously with the decline and cessation of the reaction (effervescence, etc.) and lasts for only a short time, *e.g.* a fraction of a second; the succeeding phase of gradually returning transmissivity is much more prolonged. A somewhat similar division of the recovery process into two stages can also be distinguished in living tissues, especially those with relatively long refractory periods, *e.g.* heart muscle, as already indicated. These resemblances justify the belief that a study of the conditions of recovery in the metallic system may throw light on the general nature of the recovery process in living protoplasm.

II. Phenomena of Automatic Repassivation in Iron Wires.

The following account is based on experiments with iron wires of standard composition and properties, known as "music steel wires," manufactured by the Spencer Wire Company of Worcester, Massachusetts. Wires of 1 to 1.2 mm. diameter were used (Nos. 20 and 25). The metal is bright, elastic, and highly tempered. Its behavior in nitric acid of different concentrations is definite and regular, within certain well defined limits of variation to be described below. It is important in experiments of this kind to use metal of uniform quality, for ordinary specimens of iron (*e.g.* "black iron" sheeting, soft iron wire, wire nails, etc.) are often highly variable and capricious in their manner of reaction with strong nitric acid.

I have already described briefly the appearances accompanying the activation of these wires in different dilutions of pure nitric acid.[1] Before use, the freshly cut wires are allowed to react for 15 seconds or so with dilute acid (specific gravity 1.2); they are then rinsed in water, wiped clean with a coarse cloth, and passivated by immersion in strong HNO_3 (specific gravity 1.42). The passive wires are steel bright and undergo no change if left undisturbed in dilute nitric acid (from specific gravity 1.2 up). When they are activated in dilute acid, *e.g.* by touching with zinc or scraping with glass, there is an

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immediate darkening of the bright surface (from formation of oxide), followed by active effervescence; both of these changes then spread rapidly along the whole length of the wire. A completely transmissive wire thus reacts as a whole, in a manner which in acid of a definite concentration and temperature is constant and independent of the mode of activation. The reaction continues for an indefinite time in acid below a certain concentration; this critical concentration is about 50 volumes per cent of 1.42 HNO_3 (equal to acid of 1.2 specific gravity or about 7.5 N by volume); in all stronger solutions the reaction soon comes automatically to rest and passivity is regained. In such cases the duration of the local reaction varies in a characteristic manner with the concentration of HNO_3; *e.g.*, in acid of 85 volumes per cent (about 12 N by volume) effervescence lasts (at 20°) for only a small fraction of a second, while in 55 per cent HNO_3 it continues for several seconds, at first uniformly and later declining and finally ceasing somewhat abruptly, usually after 6 to 8 seconds. During the reaction a dark brown oxide is deposited on the surface of the metal; the thickness of this coating depends on the duration of the reaction; hence immediately after spontaneous repassivation in 55 per cent acid a wire is almost black in color, while in 80 per cent acid it is only slightly bronzed. This deposit gradually dissolves away if the metal is left in the acid; it is loosely adherent and is entirely distinct from the thin, resistant, and invisible surface film which imparts passivity. The recently formed oxide-covered region furnishes a convenient indicator of the distance traveled by the activation wave in cases of partial transmission, since its boundary against less recently activated regions is always sharply defined.

On activation the potential of the metal changes suddenly from that characteristic of passive to that of active iron; in nitric acid of the above concentrations the active metal is more negative (zinc-like or anodal) by 0.7 to 0.8 volt. Hence the duration and certain other features of the reaction can be observed conveniently by the use of a voltmeter or string galvanometer. When two iron wires, bent into an appropriate shape and connected through keys to a voltmeter (preferably one with a scale of 1 to 2 volts and a central zero), are passivated by dipping into 1.42 HNO_3, and are then placed side by side a short distance apart in a flat dish containing 55 per cent acid, on

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activating one of the wires the needle of the instrument shows at once a rapid initial excursion of about 0.7 volt. During the continuation of the reaction the potential exhibits irregular rhythmical fluctuations about this point; later, as the reaction begins to decline, it decreases by degrees, finally swinging back rather abruptly to zero and a little beyond as the reaction ceases. It is characteristic that immediately after repassivation the wire is always slightly more positive than before, by a potential of 0.01 to 0.02 volt, and remains in this condition for some time; by degrees, especially if the acid is stirred, the wire resumes its original potential. The rhythmic variation of potential is an especially interesting peculiarity from the biological point of view, since rhythmicity of a similar kind is frequent in irritable living tissues, as well as in other film-covered inorganic systems, such as mercury in hydrogen peroxide. It indicates a tendency in the early stages of the reaction to an alternating formation and disruption of the passivating film; later the process of formation predominates and the film becomes continuous over the whole surface; the wire is then passive. The "after-positivity" has an analogy to the so called "positive after-variation" observed under certain conditions in nerves after stimulation;[16] from the most general point of view it is to be regarded as a polarization effect of the kind found at all polarizable electrodes.

Time Relations of Recovery of Transmissivity.—The behavior of a wire in nitric acid of 60 per cent concentration will first be described for illustration. When such a wire, after having been left undisturbed in this solution for some time (*e.g.* an hour), is touched at one end with zinc a wave of activation sweeps rapidly (at some hundred centimeters a second) over its whole length; after 1 or 2 seconds the reaction ceases and the metal becomes again passive. For some minutes after repassivation the wire is found to be incapable of transmitting activation for more than a limited distance. At the very first, touching with zinc produces only a slight local reaction; when touched 30 seconds later the local effervescence and darkening caused by the zinc are somewhat more pronounced, and the reaction spreads slowly for 2 or 3 cm. from the contact; after 1 minute there is somewhat more rapid transmission through 4 or 5 cm.; after 2

[16] Tigerstedt, C., and Donner, S., *Skand. Arch. Physiol.*, 1913, xxx, 309.

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minutes the speed is still greater and the distance has increased to 10 or 15 cm.; and after 3 minutes the wave usually travels rapidly (though less so than before the original activation) over the whole length as before. Recovery is thus a gradual process whose course can be traced by measuring the distance which an activation wave travels at successive intervals after repassivation. In the experiments about to be described the relation between the concentration of acid and the rate of recovery has been determined in this manner for solutions of nitric acid of different concentration.

The procedure has been as follows. Straight lengths of wire 20 cm. long are bent at one end into a hook shape (to facilitate handling, which is done with glass hooks and rods) and treated as already described so as to secure a uniform surface; they are then passivated by immersion in strong nitric acid (Baker's "Analyzed," specific gravity 1.42), where they may remain for an indefinite time before using. The same wires may be used over and over again in different experiments. In each experiment with a given solution several such wires, *e.g.* ten, are placed side by side in a flat-bottomed rectangular glass dish (23 by 14 cm.) containing the acid; when all the wires are in position they are activated simultaneously (or nearly so) by touching at one end with a bar of zinc; an activation wave then sweeps rapidly over each wire, which receives at the same time a coating of the brown oxide. The wires are now again passive, but at first transmit activation imperfectly, as just described. The distance traveled by the activation wave after a definite time interval of recovery is now determined by touching the ends of the wires with zinc, one by one, in succession from left to right at regular intervals. For instance, in 65 per cent acid the successive wires are activated at ½ minute intervals; a series is thus obtained showing the distances traveled after periods of recovery lasting respectively ½ minute, 1 minute, 1½ minutes, 2 minutes, etc. The distances can readily be measured with a millimeter rule; the limit of each freshly activated area is distinctly marked by the dark shade of the more recently deposited oxide; this boundary remains visible for some time after the reaction, so that if desired the measurements can be made at leisure at the conclusion of a series of experiments.

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The rate of recovery, as measured by the gain per unit of time in the traveling power of the wave, is at first gradual and then more rapid. Transmission for the full distance of 20 cm. is equivalent to transmission for an indefinite distance; it is in fact unusual for a wave that has traveled 16 cm. (at 20°) to fail to travel the whole length of any wire. Transmission for 20 cm. thus signifies full or complete transmissivity. If we define transmissivity in numerical terms as the distance in centimeters which the wave is capable of traveling along a homogeneous wire before spontaneous extinction, its values range from unity or less to infinity, the latter value signifying transmission without progressive decline in traveling power. This condition corresponds to an "all or none" behavior, or conduction without decrement, in a living conducting tissue like nerve; partial transmission is equivalent to conduction with a decrement; the numerical measure of this decrement may be defined as the reciprocal of the distance traveled before extinction.

The behavior of different wires under otherwise identical conditions is subject to considerable variation, due apparently to accidental differences in the structure of the metal in the different wires. Thus there are occasional exceptions to the rule of an increase in the distance of transmission on passing from one member of a series to the next. Table I gives the results of seventeen separate series of determinations with wires immersed in 65 volumes per cent of 1.42 acid (= about 9 N by volume). These series were all carried out on a single day, using two sets of ten wires each; these were used alternately, a determination with the one set being made while the other was undergoing repassivation in strong acid. There are thus eight series of observations with the one group and nine with the other; the wires were placed in random order in each series; *i.e.*, no care was taken to treat each wire individually as in the experiments to be described later. The figures give the distances in centimeters traveled by the activation wave along the wire when the latter is touched at one end with zinc after the intervals (since the previous complete activation) given at the head of the column. Each horizontal line gives the observations with a single series. The temperature of the acid was constant between 20 and 21°.

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It will be observed that complete recovery occupies about 4 minutes in this solution; in three series (four wires) transmission was complete after $3\frac{3}{4}$ minutes; twelve series (eighteen wires) required 4 minutes, and two $4\frac{1}{4}$ minutes. The average distance of transmission at the

TABLE I.

Concentration of HNO_3, 65 Volumes Per Cent. Temperature 20–21°C.

Series.	Distance traveled. Intervals since previous activation.									
	½ min.	1 min.	1½ min.	2 min.	2½ min.	3 min.	3½ min.	3¾ min.	4 min.	4¼ min.
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
A	2.6	4.0	4.0	7.2	8.4	8.9	15.0		Full.	
B	2.6	4.2	5.1	6.4	7.0	8.3	14.4		"	
C	2.6	2.3	3.9	4.6	7.2	8.8	15.7	Full.		
D	2.1	2.9	4.5	5.3	6.0	7.8	13.7	"		
E	1.9	2.6	3.1	5.0	7.1	8.4	13.6	"		
								(2) 15.1		
F	3.1	3.0	3.6	4.4	5.7	5.9	8.2	12.2	16.0	Full.
G	2.0	3.7	2.9	3.7	7.8	7.5	11.3	13.1	16.1	"
H	2.5	3.9	3.6	4.6	6.1	7.9	8.7	15.0	Full (2).	
I	2.1	2.9	3.9	3.6	6.2	7.4	10.3	14.7	" (2).	
J	1.7	2.4	3.9	4.6	5.7	6.7	10.4	13.6	" (2).	
K	2.5	4.3	3.5	3.7	5.6	6.1	8.3		" (3).	
L	1.5	2.5	3.7	4.4	4.2	8.4	10.5	11.9	" (2).	
M			4.5	4.7	5.4	7.5	10.7	14.0	"	
N	2.0	3.1	2.9	3.7	5.6	7.5	10.6	13.6	" (2).	
O	2.3	2.2	2.8	4.7	5.3	5.9	7.5	10.5	" (2).	
P	1.9	2.3	3.3	4.2	5.8	5.6	8.1	9.3	" (1).	
									12.2 (1).	
Q	2.2	3.0	3.3	3.0	5.8	5.9	9.8	9.9	Full (2).	
Average..	2.2	3.1	3.7	4.6	6.2	7.3	11.0	12.7	14.8	

end of each interval is given at the foot of the column. The increment (per unit of time) of transmissivity during the whole recovery period of 4 minutes is at first gradual and more rapid later. The transition from a limited to an unlimited transmissivity occupies a comparatively short interval toward the end of the period. This

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feature of the behavior has an important general significance, as will be seen below.

The same type of behavior was found in all the solutions used, which included nitric acid of the following concentrations (in volumes per cent of 1.42 HNO_3): 55, 57.5, 60, 65, 70, 75, 80, 85, 90. The time required for complete recovery of transmissivity increases rapidly with increasing concentration of acid. Table II gives the typical recovery times for different solutions at 20°. In the most concentrated solutions, 85 and 90 per cent, especially the latter, the recovery process may exhibit irregularities whose explanation is not entirely clear; and at times transmissivity remains incomplete, even

TABLE II.

Concentrations (1.42 HNO_3).	Time for complete recovery (20°).
vol. per cent	
55.0	30 – 45 sec.
57.5	60 – 70 "
60.0	90 –110 "
65.0	3½– 4 min.
70.0	6 – 7 "
75.0	9 – 10 "
80.0	12 – 13 "
85.0	14 – 16 "
90.0	18 – 20 " (or more).

after hours or days in these solutions. Apparently very strong acid renders the surface film resistant to alteration under certain conditions. Frequently, however, there is complete recovery in 90 per cent or even stronger acid; but the time required is usually 20 minutes or more. In the lower concentrations complete transmissivity always returns within the time indicated in Table II.

In their general character the measurements for the different solutions resemble those given in Table I, so that it is unnecessary to reproduce them in detail; Table III, however, gives the averages obtained for the different solutions from 57.5 per cent on.[17] In these

[17] Exact measurements with 55 per cent acid are difficult to make because the wire at the end of the reaction (which lasts 5 or 6 seconds) is so covered with oxide that the distance traveled by a later activation wave is not distinctly marked. Recovery is more rapid and the whole behavior is less regular than in the stronger solutions.

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TABLE III.

Concentration of acid.	Average distances traveled by activation wave in each solution at definite intervals after previous activation.																			
vol. per cent	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.	sec.	cm.
57.5					10	3.4	20	6.5	30	9.6	40	11.3	50	14.0 (Full transmission in 4 wires out of 19.)						
60.0			20 min.	5.3	30 min.	6.3	40 min.	7.0	50 min.	7.6	60 min.	8.9	70 min.	12.6	80 min.	12.7	90 min.	13.4 (5 full in 19.)	100 min.	16 full in 19.
65.0	½	2.2	1	3.1	1½	3.7	2	4.6	2½	6.2	3	7.3	3½	11.0	3¾	12.7 (4 full in 16.)			4	14.8 (3) (12 full in 15.)
70.0	1	1.3	2	1.5	3	2.3	4	3.8	4½	4.2	5	5.9	5½	6.7	6	10.1 (2 full in 14.)	6½	11.3 (5) (7 full in 12.)	7	All full.
75.0	1	2.0	2	2.2	3	3.0	4	3.4	5	4.3	6	5.8	7	7.7	8	10.1	9	10.8 (3) (9 full in 12.)	10	" "
80.0	2	2.0	4	2.7	6	3.6	8	5.1	10	7.3			12	10.3 (3 full in 17.)			13	All full.		
85.0	2	3.1	4	3.1	6	3.5	8	4.6	10	7.4	12	8.6	13	10.4 (5)	14	12.8 (2 full in 14.)	15	12.4 (5) (8 full in 13.)	16	5 full in 6.
90.0	2	2.5	4	2.9	6	3.8	8	4.3	10	5.4	12	6.7	14	9.5			16	11.0 (10) (6 full in 16.)	19	13.9 (2) (4 full in 6.)

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experiments the intervals between the successive activations in any series were adjusted to the time period of recovery characteristic of the solution used, as given in Table II. In each series (with a few exceptions) ten wires were used, with the procedure already described. The number of separate determinations for each of the average distances given in Table III varied from twelve to nineteen (in a few cases more), except in those cases where part of the wires had recovered

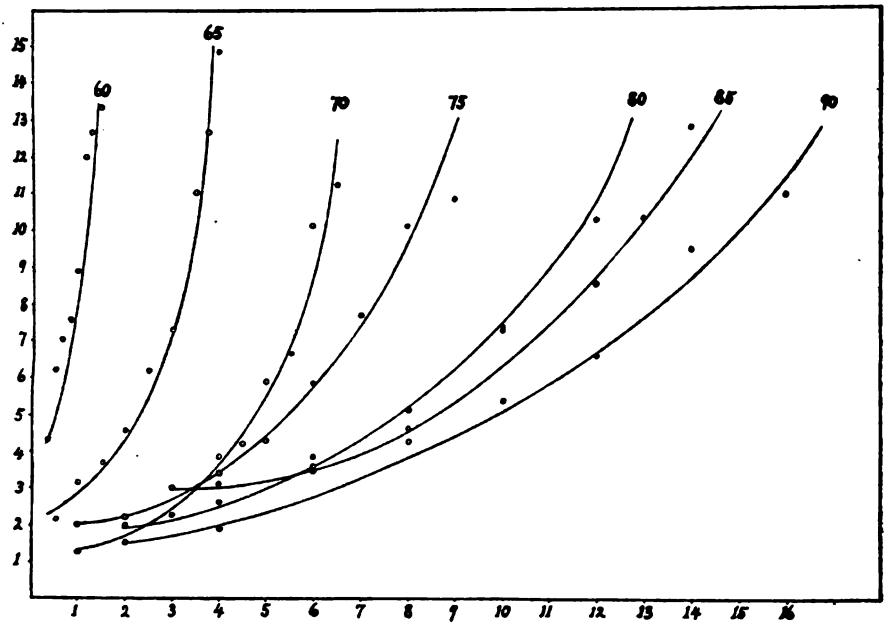

FIG. 1. Distances traveled by activation wave in different solutions of HNO_3 at different intervals after a previous complete activation in the same solution. Ordinates are distances (cm.); abscissæ are times (minutes) elapsed since the previous activation. A single curve represents the behavior in acid of the concentration indicated at the end of the curve (in volumes per cent of HNO_3, sp. gr. 1.42) at 20°. The small circles represent the averages given in Table III.

completely in the time allowed; in these cases the number of determinations is given in brackets. When plotted with distances as ordinates and times as abscissæ the data give curves of an hyperbola-like form, beginning with a horizontal and ending with a vertical course (Fig. 1).

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All these determinations were made at room temperature, and the temperature of the acid was taken at the end of each series; in all but seven of the 119 series summarized in the table the temperature was between 19 and 21°; in the others it rose to 21.5 or 22° (in one case 22.5°). The irregular fluctuations in the series have, however, nothing to do with temperature, but are to be referred chiefly to accidental variations in the surface structure of the different wires. In order to obviate this source of irregularity, as far as possible, a number of other series of determinations were made later, using a somewhat different plan. In each of these series eight wires were employed, designated A, B, C to H; each wire during passivation was kept separately in a long test-tube labeled with its letter, so that its individual behavior could be observed. Eight separate series of determinations were made as above in each of the following solutions of acid: 60, 65, 70, 75, and 80 volumes per cent, in such a manner that each wire was exposed to each solution for each of the eight periods employed in the series. For example, the times of exposure to 75 per cent HNO_3 were 2, 4, 6, 7, 8, 9, 10, and 11 minutes; in the first series with this solution the wires were arranged in the acid in the order A, B, C to H corresponding to the above times; in the second series Wire H was shifted from the end to the beginning of the series, so that it received 2 minutes exposure before reactivation, Wire A 4 minutes, and so on; in the third series the wires were in the order G, H, A, B to F, etc. In this manner a record of transmission distances was obtained for each wire with each of the eight recovery intervals allowed.

The results of these series were on the whole more regular than those of the earlier series. The wires used were of the same kind as before, but cut from a different roll (No. 25 thickness). The behavior and the recovery times showed little difference from those of the earlier series (made with No. 20 wire). Care was taken to keep the temperature constant between 19.5 and 20.5°. Table IV gives the complete record of two typical series with 65 and 80 per cent acid respectively.

These results are closely similar to those obtained in the earlier series, and no significant differences of behavior are observable in the individual wires. It is apparent that each reaction means a renewal of the reacting surface, since a thin layer of metal is dissolved each time. The irregularities of behavior are therefore probably due to

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variations in the finer grain of the metal, involving irregular local potential differences which influence the local rate of reaction and interfere somewhat with the uniformity of the reaction at different regions of the surface layer. In Fig. 2 the averages for the different solutions of these series are plotted and curves drawn as before. These averages are given in Table V.

TABLE IV.

Wire.	Distance traveled. Intervals since previous activation.							
Series 1. Concentration of HNO_3, 65 volumes per cent. Temperature 20°C.								
	1 min.	1½ min.	2 min.	2½ min.	3 min.	3½ min.	4 min.	4½ min.
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
A	4.2	4.8	4.5	5.5	8.1	10.4	11.7	Full.
B	3.2	3.2	4.1	4.0	4.9	8.3	13.3	"
C	3.2	4.4	4.6	5.7	7.5	9.1	12.9	"
D	4.0	5.2	4.4	8.3	7.4	10.2	11.5	"
E	2.3	3.5	4.2	7.1	10.5	10.3	13.5	"
F	2.8	3.9	4.9	5.9	8.4	10.5	15.1	"
G	3.8	2.5	5.2	5.9	9.6	9.9	12.0	"
H	3.1	4.5	5.0	5.5	7.3	11.0	15.5	"
Average.	3.3	4.0	4.6	6.0	8.0	10.0	13.4	
Series 2. Concentration of HNO_3, 80 volumes per cent. Temperature 20°C.								
	2 min.	4 min.	6 min.	8 min.	10 min.	12 min.	13 min.	14 min.
A	1.2	1.5	2.3	3.2	5.2	13.2	16.8	Full.
B	1.5	1.8	2.0	3.4	6.8	9.4	Full.	"
C	1.9	1.9	3.1	3.4	6.4	11.3	18.8 Full.	
D	1.7	2.7	2.7	3.3	4.5	8.9	11.6	Full.
E	1.8	2.5	2.7	3.8	6.1	7.6	14.5	"
F	1.4	2.1	2.4	4.6	6.4	11.2	9.0	"
G	1.3	2.0	3.0	4.5	7.2	9.9	12.0	10.7
H	1.5	1.6	2.6	4.1	5.4	11.2	13.4	Full.
Average.	1.5	2.0	2.6	3.8	6.0	10.3	13.7	

In this arrangement the horizontal lines give the results for the same wire with varying intervals before reactivation. Any single series is to be read along the diagonals from above down, beginning with the first column, in such a way that the letters fall in consecutive order.

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TABLE V.

HNO_3	Distance travelled.																	
	Interval since previous activation.																	
per cent	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*
60	½	2.5	¾	3.7	1	4.2	1½	5.5	1½	6.8	1¾	10.3	2	13.0 (2 full in 8.)			2½	13.1 (3) (5 full in 8.)
65	1	3.3	1½	4.0	2	4.6	2½	6.0			3	8.0	3½	10.0	4	13.4	4½	All full.
70	1	2.3	2	3.0	3	3.8	4	4.8			5	5.9	5½	7.0	6	9.4	6½	6 full in 8.
75	2	2.0	4	3.0	6	4.2	7	5.8			8	7.8	9	10.8	10	13.1 (3) (5 full in 8.)	11	All full.
80	2	1.5	4	2.0	6	2.6	8	3.8			10	6.0	12	10.3	13	13.7 (1 full.)	14	7 full in 8.

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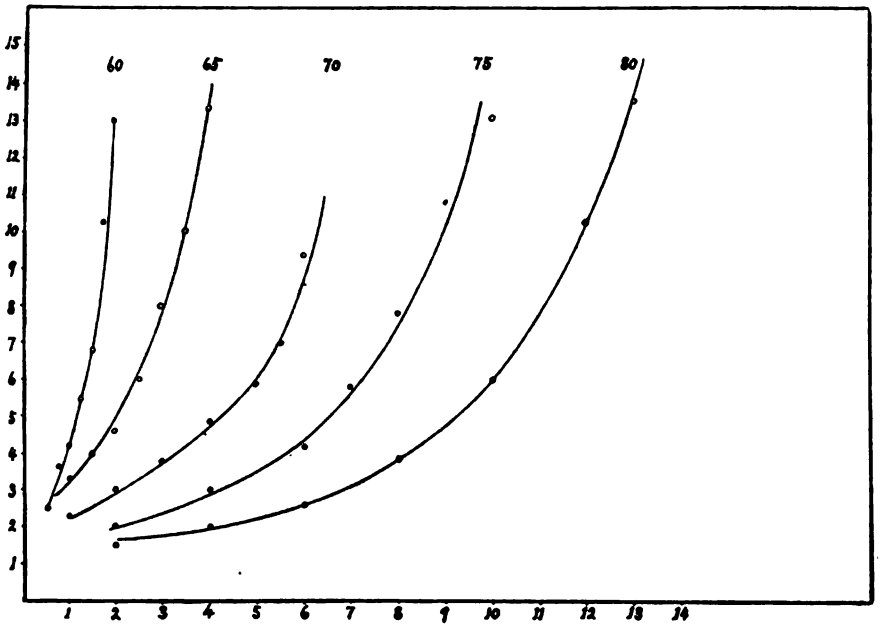

FIG. 2. Graphs for the second set of determinations with No. 25 wires. Otherwise as in Fig. 1. The circles represent the averages of Table V.

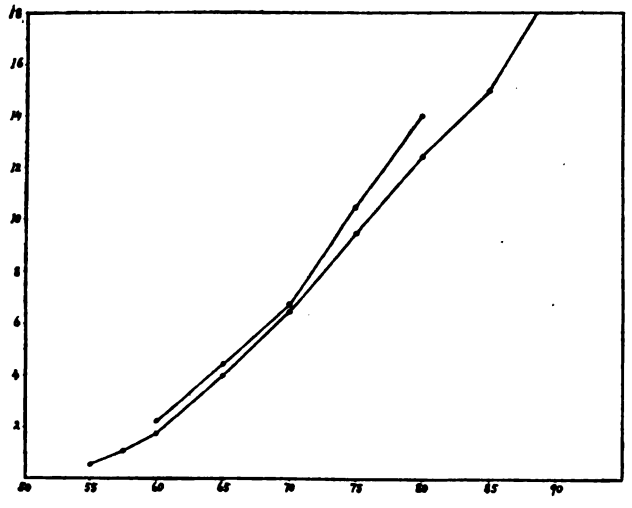

FIG. 3. Relation between complete recovery time and concentration of acid. Ordinates are times (minutes) required for recovery of complete transmissivity at 20°; abscissæ are concentrations (volumes per cent of 1.42 HNO_3). The right hand curve represents the earlier (data of Table II), the left hand the later set of determinations.

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Fig. 3 represents graphically the relation between the complete recovery time and the concentration of acid, as observed in the two sets of experiments just described. The curve approximates a straight line, indicating that the time is nearly proportional to the excess of concentration above a critical value (of about 53 to 54 per cent).

Influence of Temperature on Recovery Process.

Eight series of determinations in 65 per cent acid were made in the manner described at the temperature of about 3°, the dish containing the acid being kept cool during the experiment by immersion in snow. The results are given in Table VI.

TABLE VI.

Concentration of HNO_3, 65 Volumes Per Cent. Temperature 3°.

Wire.	Distance traveled. Intervals since previous activation.							
	2 min.	4 min.	6 min.	8 min.	10 min.	12 min.	14 min.	16 min.
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
A	4.1	4.9	6.1	9.2	8.3	19.2	16.8	Full.
B	3.8	5.8	7.0	8.8	13.4	10.3	14.6	8.6
C	4.9	9.0	10.6	11.5	9.7	17.3	13.3	16.8
D	2.2	3.3	9.1	11.2	11.4	11.9	16.2	8.4
E	1.7	5.5	11.3	5.8	14.8	10.7	9.6	19.3
F	3.1	3.9	9.3	11.4	6.4	10.1	13.1	14.8
G	3.5	3.9	7.7	8.6	11.6	11.7	Full.	Full.
H	5.5	5.2	9.8	7.5	14.7	12.6	14.4	
							13.7	
Average.	3.5	5.2	8.9	9.2	11.3	13.0	14.0	

It will be observed that recovery is four or five times slower at 3° than at 20°, only three cases out of eight showing full transmission after 16 minutes of recovery. There are also several other differences in the behavior of the wires at the two temperatures. The speed of transmission is several times slower at the low temperature; this retardation is perfectly distinct to the eye, but as yet no exact measurements of velocity have been made. The local reaction shows a less

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vigorous effervescence and lasts for 5 or 6 seconds, as compared with about 1 second at 20°; *i.e.*, the return to passivity is delayed in about the same proportion as the recovery of transmissivity. The disappearance of the deposit of dark oxide is also much more gradual. In partly recovered wires the retardation in the speed of the activation wave as it nears its stopping point is more distinct. There are also a larger number of apparently arbitrary irregularities in the distances

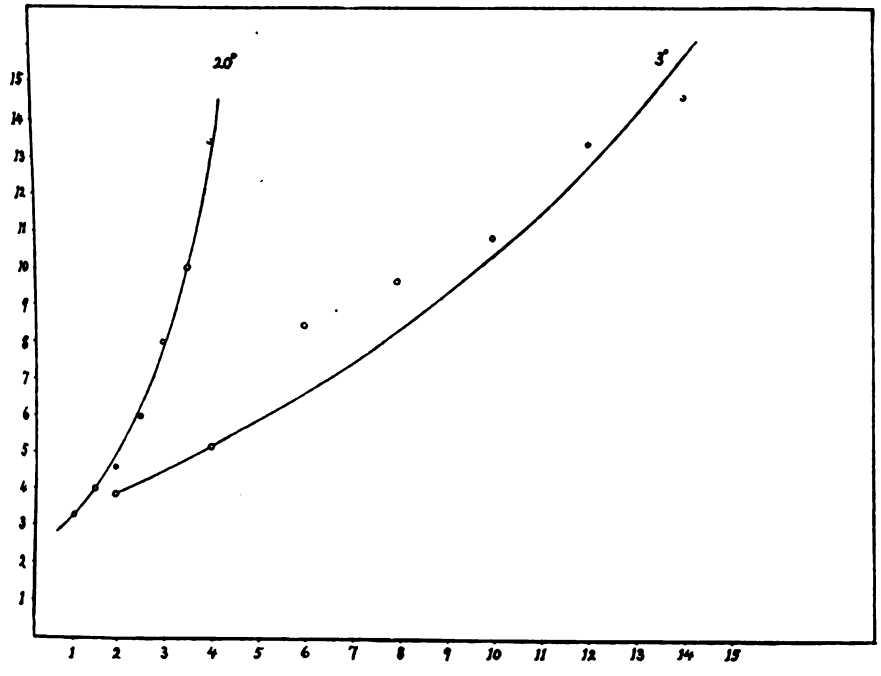

FIG. 4. Curves showing rates of recovery in 65 per cent acid at the two temperatures, 20° (Table V), and 3° (Table VI). Ordinates are distances (cm.), abscissæ, times as before.

traveled; thus Wires B and D transmitted for only about 8.5 cm. after 16 minutes in the acid, and other reversals in the expected order of the figures are seen in Wires A, C, E, F, and H. Apparently the conditions (irregular local circuits, etc.) which interfere with transmission are more effective when the local chemical processes are slower. The points representing the average transmissions at the several in-

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tervals group themselves with fair regularity into a curve of the usual type but of more gradual slope (Fig. 4).

The characteristic temperature coefficient shown (Q_{10} = 2–3) is evidently an expression of the dependence of repassivation and recovery of transmissivity upon chemical reactions occurring at the metallic surface. These reactions rebuild the surface film and restore it to its original condition. The temperature coefficients of the duration of the refractory period in cardiac and skeletal muscle and in nerve are closely similar.[15] This resemblance may be regarded as a further confirmation of the view that in the latter case also the recovery of irritability and transmissivity is dependent primarily upon the chemical processes concerned in the restoration of the protoplasmic surface films.

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THE RECOVERY OF TRANSMISSIVITY IN PASSIVE IRON WIRES AS A MODEL OF RECOVERY PROCESSES IN IRRITABLE LIVING SYSTEMS.

PART II.

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Theoretical Considerations.

The consideration of why transmission is only partial during the early period after repassivation must take account of a number of factors whose precise mode of action is not clear in all cases. It is evident that the return to the passive state implies redeposition of a continuous surface layer of oxidation product; it is also evident from the behavior of the newly passivated wire that when first deposited this layer is in a different chemical and physical condition from that which it afterwards attains when complete transmissivity is reestablished. The question is what kind of change occurs in the surface layer during the period of progressive recovery; *e.g.*, in 70 per cent HNO_3 during the first 7 or 8 minutes after the spontaneous return of passivity.

Several peculiarities are to be noted in the character of the activation wave at the successive intervals. There is an evident correlation between its speed and the distance which it travels. At first the local reaction spreads slowly and for only a short distance; at each successive trial the wave travels faster and advances farther; eventually it travels for an indefinite distance at a high speed (several hundred centimeters per second). It is also noticeable that an activation wave which comes spontaneously to rest after traveling some distance is visibly retarded through the last 2 or 3 cm. of its path (though not

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evidently retarded earlier);[1] this retardation is more readily seen at low temperatures (3°C.), and in general in slowly moving than in rapidly moving waves.

It is well known that the advance of the wave is due to local cathodic reduction at the boundary region between the active and the passive areas of the metal, the passive area being cathodal.[2] The diagram (Fig. 1) illustrates the conditions. The active area is anodal and the adjoining passive area cathodal. The local intensity of the current passing between the solution and the passive metal at any point decreases with increase in the distance from the boundary line X, because of the increase in the electrical resistance of the circuit which includes the point under consideration. This resistance depends almost entirely upon the length and the specific electrical resistance

FIG. 1. Indicating the conditions of the local circuit at the boundary between the active and the passive areas; the direction of the current (positive stream) is indicated by the arrows. The active region (shaded) is anodal, the passive cathodal. See text.

of the column of electrolyte between the point in question and the boundary line. The local reducing action, which is a function of the local intensity of the current, thus decreases as the distance from the boundary increases, *i.e.* in the order A < B < C, and beyond a certain distance from the boundary (*e.g.* XY) it will be insufficient to

[1] Adrian (Adrian, E. D., *J. Physiol.*, 1914, xlviii, 53) finds no evidence of change in the velocity of the excitation wave in the nerve as it passes along a region of decrement; but, as he himself points out, his observations do not refer to the rate of conduction immediately before the extinction of the wave. He concludes that "if the rate of conduction is ever affected by the size of the disturbance it can be only when the disturbance is so small as to be on the verge of extinction." In the passive wire also the rate of transmission does not undergo evident retardation until immediately before extinction.

[2] *Cf.* Bennett, C. W., and Burnham, W. S., *J. Phys. Chem.*, 1917, xxi, 107. For the resemblances to protoplasmic transmission, *cf.* Lillie, R. S., *Am. J. Physiol.*, 1916, xli, 126; *Science*, 1918, xlviii, 51; 1919, l, 259, 416; *J. Phys. Chem.*, 1920. xxiv, 165.

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effect reduction; during transmission, therefore, we are to assume that the removal of the film by cathodic reduction is continually in progress through a certain distance in advance of the boundary. The rate of this reducing action, and the distance from the boundary through which it is effective (*i.e.* the length of XY) are the two chief factors determining the speed of propagation.

It seems probable that in the decrement type of transmission the removal of the passivating film by cathodic reduction in the region XY adjoining the boundary is everywhere incomplete, and that the progressive decline in the traveling power of the activation wave is thus to be explained. In the case just considered, however, in which the local reduction is assumed to be complete, with consequent exposure of a free surface of metallic or "active" iron over the entire area XY, there will inevitably be complete transmission; *i.e.*, the activation wave, once set up, will travel through an indefinite distance, since then the area secondarily activated by the local current will have the same properties as the original active area; accordingly the current conditions at the boundary of this new active area will be the same as at the original area, and these conditions will be repeated at every new boundary as the active region extends. With exact repetition of the same reducing effect at the cathodal (*i.e.* passive) area adjoining each new boundary as it is formed, there is nothing to prevent transmission through an indefinite distance. In transmission with a decrement, however, it is evident that the chemical effect at each new boundary is somewhat less than before, as shown by the progressive decline in the traveling power of the activation wave. If, as the above hypothesis holds, the removal of the passivating film near the boundary is in this case only partial, this partially activated area will not reach the full anodal potential characteristic of a freely exposed iron surface; and the reducing effect at the passive area adjoining will be less than at the original active area; *i.e.*, there will be a progressive decline in the reducing power of the local circuit as the active area advances. Eventually, when the boundary of the active region has advanced for a certain distance from the starting point, the P.D. of the local circuit will fall below the critical value (*i.e.* the decomposition voltage) required for appreciable reduction; the advance of the active region will then cease, and the line of cessation will be

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sharply defined. According to this hypothesis, the P.D. across the active-passive boundary decreases progressively as the activation wave passes along a region of decrement. When the rate of this decrease is gradual, there will be transmission through a relatively great distance before the wave comes to rest; when it is rapid, transmission will be correspondingly limited.

This hypothesis is also consistent with the fact that the return of transmissivity is more rapid in weak than in strong acid, its rate being approximately inversely proportional to the excess of concentration above a certain critical level (about 53 to 54 volumes per cent).[3] Two chief factors may be distinguished in the chemical part of the passivating process, *i.e.* in the reaction which reforms the passivating surface layer of oxide: (1) the general oxidative action of the solution, which is the more intense the higher the concentration of HNO_3; and (2) the electrochemical oxidative action at the anodal region of the local active-passive circuit. This anodal area, *i.e.* the active region of the metal, is automatically subjected to the oxidizing—and hence passivating—influence resident at every anode. The difference in the rate of repassivation is an index of the energy of the local oxidizing process; in acid of 50 volumes per cent (about 7.5 N) or lower this is not sufficient to rebuild a stable surface film, hence the metal continues to dissolve in this solution; in 55 per cent acid the oxidative action is intense enough to deposit a permanent film in 5 or 6 seconds (at 20°); in 60 per cent acid the rate of deposit (*i.e.* of repassivation) is several times more rapid; and at higher concentrations still more rapid. The low degree of transmissivity found immediately after repassivation suggests that the passivating film when first deposited is relatively thick (or relatively dense or otherwise resistant to alteration) in comparison with what it becomes later, when the state of transmissivity without decrement is reached. This view would imply that the quantity of passivating material deposited during the reaction

[3] The formula $\frac{C - 54}{t} = \text{constant}$ expresses fairly well the relation of the total recovery time to the concentration of the acid, where C is the concentration and t the recovery time. 53 to 54 per cent is the critical concentration; and the time required for the return of complete transmissivity is about proportional to the excess of concentration above this value.

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is closely proportional to the excess of concentration of the acid above a certain critical limit (of about 53 to 54 per cent).[4] Hence in strong acid the film at its first deposition has a thickness (or a structure) such that the relative quantity removed by the cathodic reduction at the active-passive boundary when the metal is locally activated is less than in weaker acid. The decrement of transmission is hence from the first steeper in the strong acid, and a greater time is required for the removal of the surplus film material (by the solvent action of the acid) until it attains a degree of thinness permitting of rapid and complete removal under the influence of the local circuit.

The important fact from the point of view of the physiological comparison is that after the decrement stage has passed the local reaction is complete and does not admit of gradations. Transmissivity is then complete and the wire as a whole exhibits an "all or none" type of response. This stage is a permanent one, as long as the metal remains undisturbed in the acid, and apparently it corresponds to a condition of minimal thickness of film. Judging from the analogy with adsorption processes in general, it seems probable that at this stage the film is only 1 molecule in thickness.[5] It is evident that with a film of this thickness there would be no possibility of gradation in the local action; either it would be complete or would not occur. Uniform and complete action at the different areas of the surface in a transmitting wire would thus be necessitated, with the result that the activation wave would be transmitted for an unlimited distance with a uniform velocity whose exact degree would be determined by the local rate of reduction of the film.

These considerations lead to the conclusion that the condition reached by the passivating film when complete transmissivity is regained is one in which its material is spread out in a uniform layer of 1 molecule in thickness. In this respect it corresponds in structure to films of oil or fatty acid which have spread over the surface of water to an equilibrium stage, as in Langmuir's experiments.[6] The precise composition and structure of the film do not concern us at present;

[4] *Cf.* Lillie, R. S., *J. Gen. Physiol.*, 1920–21, iii, 119, Table II; and 125, Fig. 3.
[5] *Cf.* Freundlich, H., Kapillarchemie, Leipsic, 1909, 278.
[6] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

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it is an oxidation product and is usually regarded as a higher oxide of iron.[7]

The question remains why the film persists for an indefinite period with unaltered properties, even in acid of a strength lower than that required to passivate. Why should not the film undergo still further solution and eventually lose its continuity and break down? In point of fact, the passivating film is unstable except in solutions possessing considerable oxidizing power;[8] its preservation thus appears to depend upon a more or less continuous process of slow oxidation; this process exercises what may be described as a regulative control over its local variations of composition or thickness. That this is the case is indicated by the general fact that passivity disappears spontaneously in pure water and in solutions of salts and other substances, except those having strong oxidizing properties. In the latter case there is evidence that local interruptions of the film (unless too extensive) are automatically repaired by local oxidative action. This may occur even in solutions whose oxidative properties are insufficiently intense to repassivate a completely activated wire. The case of nitric acid of specific gravity 1.20 or less will illustrate; in this solution an active wire continues to react until it is completely dissolved, but a passive wire remains unaltered indefinitely. I have already described how a film partially destroyed by brief immersion in M/1,200 NaCl is restored to its original state by brief immersion in 1.20 HNO_3.[9] A similar restoration after partial mechanical removal of the film is seen when the passive wire, while immersed in 1.20 HNO_3, is scraped locally with a piece of glass; in order to secure activation by this means several scrapes in rapid succession are as a rule required; if the interval between successive scrapes is lengthened this characteristic summation effect is less readily obtained. When a wire which is thus treated is connected through a voltmeter with another wire (*e.g.* of passive iron or platinum) serving as an indifferent electrode, there is seen at each scrape a slight temporary excursion of the needle

[7] Bennett and Burnham.[2] Bennett, C. W., and Burnham, W. S., *Tr. Am. Electrochem. Soc.*, 1916, xxix, 217. Langmuir (Langmuir, I., *Tr. Am. Electrochem. Soc.*, 1916, xxix, 260), however, has a somewhat different conception.

[8] For examples of this behavior *cf.* Lillie, R. S., *Science*, 1919, l, 259, 416.

[9] Lillie, R. S., *Science*, 1919, l, 259.

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of the instrument, the scraped wire becoming more negative, indicating the formation of an anodal area. The range of this excursion is increased by a rapid succession of scrapes, and when a critical point is overpassed a propagated activation wave is started and the whole wire is activated. The reaction thus produced is permanent in acid of specific gravity 1.20 or lower; with higher concentrations it is temporary, as already described. The return of the potential to zero after its disturbance by the local destruction of the film evidently indicates the formation of a new film at the scraped area. Any local disruption of the film renders that area anodal, by exposing the underlying iron, and hence subjects it automatically to the anodal oxidizing influence. This at once reforms the film, unless the free metal is exposed over more than a certain critical area, in which case the active area *spreads* instead of becoming obliterated and the whole wire becomes active. Slight local disruptions, however, are at once repaired and the continuity of the film is in this manner automatically preserved. Hence the passive state is a stable one in solutions of sufficient oxidizing power.

It should be noted that this automatic tendency for the film to reform explains why the rapid removal of a considerable area of film is necessary for mechanical activation, and also the need for a brief interval between successive scrapes in the summation phenomena just described. It also explains the greater effectiveness of rapidly increasing as compared with slowly increasing currents in electrical activation.[10] Otherwise the film may be reformed as rapidly as it is removed, and the local effect remain insufficient to start a wave of activation.

We infer therefore that the condition of equilibrium which the passive metal reaches eventually when immersed in nitric acid is one in which a thin continuous layer of oxygen compound 1 molecule in thickness covers its entire surface. There is nothing to prevent a thicker layer from being thinned by solution until it reaches this limit, but any further removal is prevented by the automatic regulatory reaction just described. In this sense the preservation of the passivating film in an oxidizing solution is the expression of a "dy-

[10] Lillie, R. S., *Science*, 1918, xlviii, 57.

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namic" rather than a "static" type of equilibrium; and there is seen an interesting analogy to the process by which a protoplasmic structure such as the plasma membrane maintains intact its structural continuity and dependent properties (semipermeability, polarizability, etc.) during life.

The brief duration of the period of activity in a wire activated in stronger solutions of nitric acid indicates a rapid reformation of the film, implying a correspondingly active local oxidation. The frequent failure of transmission in solutions stronger than 85 per cent (of 1.42 HNO_3) probably indicates a too intense oxidizing action, which interferes with the local reduction on which transmission depends. This view is confirmed by the fact, repeatedly verified throughout the present investigation, that mechanical activation is more readily induced in weaker than in stronger acid. In general, activation is favored by conditions that promote reduction (like making the metal cathodal) and hindered by conditions of the reverse type. This is illustrated by the following experiment. When two passive iron wires are placed side by side about 2 cm. apart in 60 per cent HNO_3 and connected through a key with the poles of a single Edison cell (about 0.9 volt), it is usually found, on closing the circuit, that the current is insufficient to activate the cathodal wire. During the flow of the current, however, this wire is more readily activated by scraping with a glass slide than while the current is not flowing; conversely, the anodal wire, during the flow of the current, is much more resistant to mechanical activation than before. But immediately after breaking the current the anodal wire becomes temporarily *more* reactive than normally—an effect due probably to its being now the cathode of the reverse or polarization current. Electrical activation is similarly modified by the passage of a constant current between a passive wire and the acid in which it is immersed; for example, while a current from five cells (about 4.5 volts) was passing through a passive wire anode immersed in 70 per cent HNO_3, touching with zinc caused a local activation which was conducted for only a few centimeters from the region of contact; after the current was broken the wave was transmitted as usual for the whole length of the wire. All the above effects indicate how closely the behavior of passive wires depends upon the condition of the surface film and upon the rate and character of the chemical

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processes occurring at the surface of the metal. The parallelism of these physical effects to the phenomena of electrotonus in irritable living tissues is evident. Either facilitation or hindrance of the processes of physiological activation and conduction may result by altering the electrical polarization of the cell surface through a constant current, the effect varying according to the degree and orientation of the polarizing influence; and the above experiments show that the same is true of the film-covered metallic system.

It is remarkable how closely the behavior of passive iron wires during the period immediately following repassivation simulates that of partially narcotized or asphyxiated nerves, or of nerves in which some other kind of "block" is established. The "decrement" type of transmission characteristic of such wires is also shown by normal or completely transmissive passive wires under certain special conditions, *e.g.* in the neighborhood of a piece of platinum or other noble metal in close contact with the wire. Such contact invariably retards or prevents the passage of an activation wave started in another region. The explanation of this interference is simple; platinum is nobler in the electrochemical scale than passive iron; hence near the contact there exists a local circuit in which the iron is anodal, a condition which, as already described, interferes with activation and transmission. The following experiment will illustrate. If a piece of platinum wire or foil is placed across the middle region of a passive wire immersed in HNO_3 (of 60 to 80 per cent) and pressed into close contact by means of a glass rod, an activation wave started at one end of the wire is typically blocked at the region of contact and fails to enter the region beyond. The stoppage of the wave is not abrupt, but occurs with a progressive retardation which is plainly visible at a distance of 1 or 2 cm. from the contact. With an insufficiently close contact (*i.e.* too small a contact area) the block may not be complete; in such a case the activation wave is often observed to undergo marked retardation—at times almost stopping—at the contact; but after passing this region it regains its former speed, at first by a visible acceleration, and travels in the usual manner to the end of the wire. The parallel to a partial block in a nerve, due to local mechanical or chemical treatment, is evident; in this case also there is retardation in the altered region (or region of decrement), and, if the excitation

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wave emerges from this region into the normal region beyond, it there regains its former velocity and other characteristics.[11] The conditions in the passive wire suggest that a block resulting from local injury or constriction in a nerve is due mainly to the influence of the local current (or injury current) between the altered and the unaltered regions—just as the platinum blocks the activation wave in a passive wire by interposing in its path a local circuit in which the iron is anodal and hence resistant to activation. In the metallic system partial compensation of the current of the approaching active-passive circuit by means of the current of the local Pt-Fe circuit is probably also a factor in the extinction of the wave. Analogous conditions may be presumed to exist in the living tissue.

A fully recovered passive wire exhibits an "all or none" type of behavior, in this respect also resembling a normal nerve; while any region of a passive wire which has only partially recovered is one in which an activation wave is conducted with a decrement. Such a local region of decrement may be produced at any desired position in a wire which is elsewhere completely transmissive by the simple method of touching the wire locally with a piece of zinc at an appropriate interval after the passage of a normal activation wave. A local region of temporary activity is thus produced, beyond whose limits the wire remains unaffected. This local region continues to conduct with a decrement at a time when the remainder of the wire has completely recovered. For example, in 65 per cent acid the recovery of complete transmissivity usually occupies between 4 and 5 minutes at 20°; at 2 minutes after a previous complete activation a new wave travels (on an average) for about 4 cm. from the point of contact. If at this time one touches the wire at a central point with zinc, there is formed a sharply defined newly activated region about 8 cm. in length, which 3 minutes later, at a time when the rest of the wire has recovered completely, still conducts with a decrement. An activation wave which is then started at one end of the wire will travel to this region of decrement and penetrate the latter (usually with a visible progressive retardation) for a variable distance of some centimeters. In a certain proportion of cases, if the time relations are

[11] Adrian, E. D., *J. Physiol.*, 1913, xlv, 389.

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properly adjusted, the penetration is complete; and the wave, on emerging into the completely transmissive region beyond, recovers its former velocity and continues to the end of the wire. An activation wave will in this manner penetrate *two* regions of decrement separated by an interval of complete transmissivity, while it will be blocked by a single continuous region of a length equal to the sum of the other two. Such an experiment is closely comparable with Adrian's well known experiment in nerve.[11]

It is, however, difficult, on account of the variable behavior of different iron wires, to reproduce this result at will with the above method. The following procedure is more satisfactory and gives the

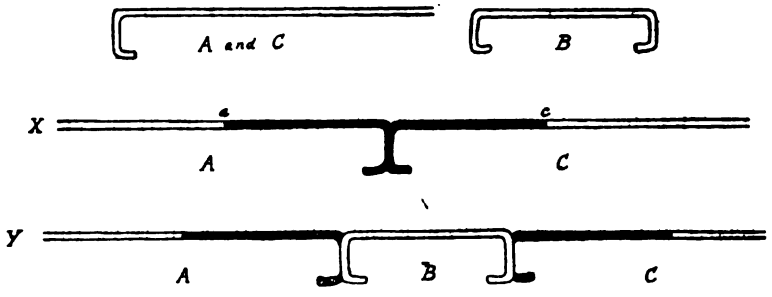

FIG. 2. Form and arrangement of wires in the experiment showing the difference in transmissivity between continuous and discontinuous decrement areas. See text.

result just described in a large proportion of cases. Instead of producing as above a region of decrement in a single continuous wire, three separate lengths of wire are used, bent in one plane as in the diagram (Fig. 2). Good contact between any two wires can be secured by resting one bent end across the other so that the right-angled pieces are in contact and the long portions in a straight line. In such an arrangement with two completely transmissive wires an activation wave travels from the end of one wire to the end of the other without appreciable retardation at the junction, and the two wires behave like a single wire of double length. The experiment is performed as follows. The three lengths of wire (first rubbed bright and clean) are passivated in the usual manner and transferred to a dish containing acid of 65 to 70 per cent strength; they are now in a com-

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pletely transmissive condition and conduct activation rapidly from end to end. A region of decrement 4 cm. long is now made in each of the long wires A and C as indicated; this is done by pressing a piece of platinum foil against the wire at a point 4 cm. from the bent end and then touching this end with zinc; the wire is thus activated from its extremity to the edge of the platinum contact, and this recently activated stretch (the shaded area in Fig. 2) acts as a region of decrement for a period of several minutes. Wire B is used as a bridge between wires A and C as indicated; it is left unchanged; *i.e.*, is completely transmissive. When wires A and C are placed end to end, as in Fig. 2, X, there is a continuous region of decrement *ac* 8 cm. long; at a certain interval after the previous activation recovery in this stretch has advanced so far that a wave started at the free end of either wire will usually traverse *ac* for a distance of 6 to 7 cm.; *i.e.*, will cross the junction but fail to penetrate the whole region of decrement. The time required to reach this condition in 70 per cent acid is usually 6 to 7 minutes. The effect of interposing a completely transmissive region between wires A and C—after allowing the same time for recovery as in the previous experiment, so as to have the same degree of decrement as before—is obtained by uniting wires A and C not directly but through the bridge of completely transmissive wire B as indicated in Fig. 2, Y. In this case the wave, started at the free end of A, typically passes along the whole three wires to the extremity of C. In favorable experiments the retardation in the decrement region of A and the recovery of speed in the bridge B are plainly visible; the activation wave on entering C is then able to pass entirely through its decrement area to the end.

It is evident that the activation wave loses steadily in penetrative power—or what might be called intensity[12]—as it passes along the recently activated stretch or region of decrement; and regains this power when it enters the completely transmissive stretch beyond. This is the type of behavior also observed in a nerve fiber, as Adrian[11] has shown. In the wire the variations in transmissive power are

[12] Lucas points out that the only definite numerical measure, at present known, of the "intensity" of a nerve impulse is the distance which it can travel along a region of known decrement (Lucas, K., Conduction of the nervous impulse, New York, 1917, Chapter II).

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dependent on variations in the condition of the surface film; and it is to be inferred, if the general conditions determining transmission are similar in the living system and the metallic model, that the same is true of the conducting protoplasmic strand or nerve fiber; *i.e.*, that the decrement-producing anesthetic acts by modifying the condition of the protoplasmic surface film.[13]

As Lucas and Adrian have pointed out, conduction with a decrement is undoubtedly a physiologically normal phenomenon in many regions of the central nervous system, as well as in the myoneural junctions and other nerve endings and synapses.[14] Whether a conducting element or combination of elements in the living organism transmits excitation in the "all or none" manner or with a decrement depends not only upon its special peculiarities of structure or organization but also upon its physiological condition—state of metabolism, fatigue, etc.—at the time. There is much independent evidence that protoplasmic excitation and transmission are in general dependent on local and transmitted alterations of the protoplasmic surface films or plasma membranes under the influence of the local bioelectric currents accompanying activity. If this is true, conditions changing the properties of these films must influence the whole behavior of the protoplasmic system. The phenomena of anesthesia in particular seem to afford many instances of this kind of correlation.[13]

In living animals the time relations of the recovery process in the different irritable cells and tissues vary widely, and they usually exhibit a close correlation with the time relations of the respective excitation processes. In most cases recovery is more rapid in the living tissue than in the passive iron model; yet this is not always the case. In the photoreceptors of Mollusca recovery may require several minutes, and in the smooth muscle of the mammalian ureter Engelmann found under some conditions imperfect transmission for 15 seconds or more after the passage of a contraction wave.[15] The

[13] The evidence that the anesthetic acts primarily upon the plasma membrane of cells is summarized in my review, The theory of anaesthesia (Lillie, R. S., *Biol. Bull.*, 1916, xxx, 352).

[14] Lucas, K., Conduction of the nervous impulse, New York, 1917, Chapters X and XIII.

[15] Engelmann, T. W., *Arch. ges. Physiol.*, 1869, ii, 271.

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high velocity of the recovery process in highly irritable tissues like nerve, where the relative refractory period lasts for only a few thousandths of a second, must be referred to special peculiarities of structure and metabolism whose detailed nature is entirely unknown at present.

SUMMARY.

1. Passive iron (steel) wires, when activated after prolonged immersion in nitric acid of 55 to 90 per cent concentration (volumes per cent of HNO_3, specific gravity 1.42) revert spontaneously to the passive state, after a temporary reaction which is transmitted rapidly over the whole length of wire. The duration of this reaction at any region decreases rapidly with increase in the concentration above a certain critical limit of 52 to 54 per cent. In weaker acid (50 per cent and lower) the reaction continues uninterruptedly until all the metal is dissolved.

2. Immediately after this automatic repassivation the wire fails to transmit activation through more than a short distance (1 to 2 cm.); if left undisturbed in the acid it recovers by degrees its power of transmission (as measured by the distance traveled by an activation wave), at first slowly, then more rapidly; eventually, after an interval varying with the concentration of acid and the temperature, the activation wave is transmitted through an indefinite distance as before.

3. The return of complete transmissivity in 55 per cent acid occupies less than a minute (at 20°); in stronger acid it is more gradual, requiring in 90 per cent acid 20 minutes or more. This "complete recovery time" is nearly proportional to the excess of concentration of acid above the limiting value of 53 to 54 per cent.

4. In a given solution of acid the rate of recovery exhibits a temperature coefficient closely similar to that of most chemical reactions at this temperature (3–20°), and also to that of the rate of recovery (refractory period) of irritable living tissues after stimulation (Q_{10} = about 3).

5. Two definite phases are distinguishable in the recovery process: (1) the redeposition of the continuous passivating surface layer (of oxide or oxygen compound); and (2) the progressive change of the newly passivated wire from the state of incomplete to that of complete

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transmissivity. The former phase is of brief duration and is indicated by a sudden change in the electrical potential of the wire, from that of active to that of passive iron; this phase is succeeded by the second and more prolonged period during which the passivating layer undergoes the progressive alteration associated with the recovery of transmissivity. This alteration appears to consist in a progressive thinning of the passivating film until a minimal thickness of (probably 1 molecule) is attained. Further thinning is prevented by local electrochemical oxidation.

6. The phenomena of partial or limited transmission during the second phase of the recovery process show a close correspondence with the phenomena of conduction with decrement in irritable living tissues such as nerve. Other analogies with the behavior of irritable tissues (threshold phenomena, distinction between "local" and "propagated" effects, summation, effects resembling electrotonus) are described.

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A THEORY OF INJURY AND RECOVERY.

I. Experiments with Pure Salts.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, September 14, 1920.)

Previous researches[1] indicate that the process of death conforms to the laws of chemical dynamics and that further investigation of this subject may throw light on the fundamental mechanism of normal life processes. The present series of papers continues these studies and deals with the recovery of cells after exposure to toxic solutions.

When the marine alga *Laminaria* is transferred from sea water to a solution of sodium chloride (of the same conductivity as the sea water) its electrical resistance falls steadily until it reaches a stationary condition, which indicates death. If the tissue is replaced in sea water before this point is reached we observe that the resistance rises; this may be called recovery. In earlier stages of the death process recovery may be complete (*i.e.* the normal resistance may be regained) but this is not the case in the later stages. This is evident from Fig. 1, which shows the death curve in a solution of NaCl and recovery curves after various periods of exposure to the solution.[2]

If in place of sodium chloride we employ calcium chloride and various mixtures of these salts, varying the times of exposure, and sometimes transferring the plant from one of these solutions to another, instead of replacing it in sea water, a very complicated set of curves is obtained. It is of interest to find that these may be predicted

[1] Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533; *J. Gen. Physiol.*, 1920–21, iii, 15.

[2] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 15. The recovery curves rise to definite levels at which they may remain for days under favorable conditions. Often, however, there is a gradual decline which may be more rapid than that of the control.

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with considerable accuracy by assuming that the electrical resistance is proportional to the amount of a substance in the cell which increases or decreases according to the relative proportion of salts in the external solution.

We assume that this substance (M) is formed and decomposed by a series of reactions[3] of the type[4]

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$$

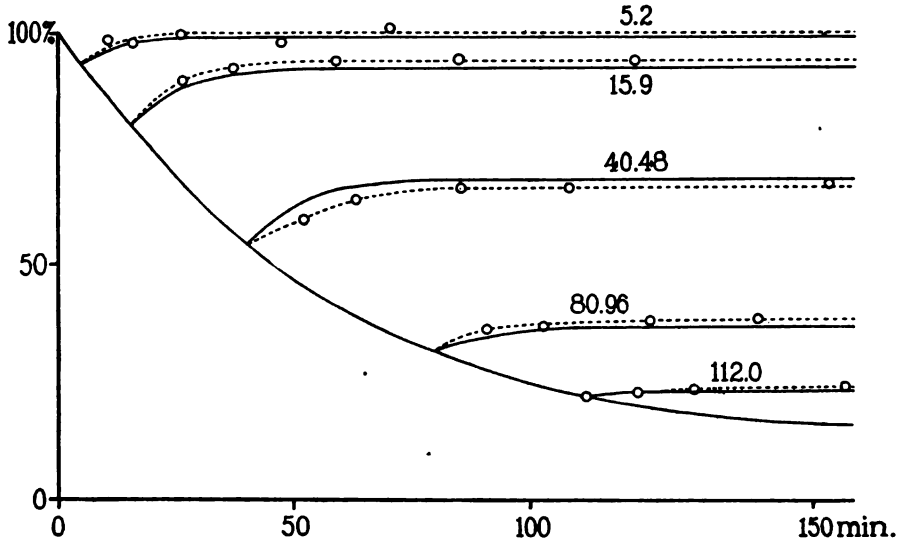

FIG. 1. Curves showing the fall of electrical resistance of *Laminaria agardhii* in 0.52 M NaCl (descending curve) and recovery in sea water (ascending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) of the solution of NaCl.

In the recovery curves the experimental results are shown by dotted lines, the calculated results by the unbroken lines (the curves are extended beyond the last observed point here shown because of later observations which are not shown in the figure).

The observed points represent the average of eight or more experiments; probable error of the mean less than 10 per cent of the mean.

[3] These are regarded as irreversible or practically so.

[4] It is assumed that O is present in relatively large amount so that it may be regarded as practically constant despite the fact that it very slowly decomposes to furnish S, A, M, and B.

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and that in sea water these processes are in equilibrium (so that the amount of M remains constant) but that when the tissue is transferred to a solution of sodium chloride M is decomposed faster than it is formed and hence the resistance falls. On replacing the tissue in sea water, M is formed more rapidly than it is decomposed and in consequence the resistance rises.

Let us assume that when the tissue is transferred from sea water to the solution of NaCl the reactions $O \rightarrow S \rightarrow A$ cease and that the velocity constant K_A of the reaction $A \rightarrow M$ increases from 0.0036 to 0.0180 while the velocity constant K_M of the reaction $M \rightarrow B$ increases from 0.1080 to 0.540. We may then calculate the resistance in the solution of NaCl after any length of exposure by means of the formula[5]

$$\text{Resistance} = 2{,}700\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 90\, e^{-K_M T} + 10 \qquad (1)$$

in which T is the time of exposure in minutes, and e is the basis of natural logarithms. 10 is added in the formula because the base line is taken as 10 (not as 0) for the reason that the resistance sinks to 10 (as shown in Fig. 1) when the tissue dies.

We assume that when the tissue is replaced in sea water the reactions $O \rightarrow S \rightarrow A$ recommence and that the values of K_A and K_M become 0.0036 and 0.1080 respectively, while the other velocity constants likewise acquire the values which they normally have in sea water. Under these conditions M will be formed faster than it is decomposed and the resistance will rise.

The fact that the rise does not reach as high a level after a long exposure as after a short one indicates that during the exposure O gradually diminishes; we assume that this takes place by the reactions

$$N \rightarrow O \rightarrow P$$

We likewise assume that during exposure to the solution of NaCl the amount of S changes by means of the reactions

$$R \rightarrow S \rightarrow T$$

and that on transferring to sea water S is rapidly converted into A.

[5] For an explanation of the formula see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533. The constants 8.853 and 0.2951 are here multiplied by 305, becoming 2,700 and 90 respectively.

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In order to calculate the rate of recovery we find by trial the most satisfactory values of the velocity constants. The values thus found are given in Table I.

TABLE I.

Velocity Constants.

Reaction.	Velocity constant.	Value at 15°C. in	
		NaCl	$CaCl_2$
$N \rightarrow O$	K_N	0.03	0.0045
$O \rightarrow P$	K_O	0.0297	0.004455
$R \rightarrow S$	K_R	0.04998	0.0145
$S \rightarrow T$	K_S	0.02856	0.007
$A \rightarrow M$	K_A	0.018	0.0018
$M \rightarrow B$	K_M	0.540	0.0295

As an example of the method of calculation we may take the case of tissue exposed for 15 minutes to a solution of 0.52 M NaCl at 17°C. The net[6] resistance in sea water at the start was 960 ohms; in the course of 15 minutes in the solution of NaCl it fell to 775 ohms, which is 80.69 per cent of the original resistance.[7] The fall of resistance is a little more rapid than in the "standard curve" obtained in a previous investigation.[8] If we assume that this is due to the difference in temperature (these measurements were made at 17°C. while those on which the standard curve is based were obtained at 15°C.) we may introduce a correction by multiplying the abscissa by the factor[9] 1.06, which makes it 15.9 minutes, and causes it to agree with the standard curve. All the abscissæ are multiplied by the same factor.[10] The

[6] The net resistance is that of the tissue itself, obtained by subtracting the resistance of the apparatus from the total resistance.

[7] For convenience all results are expressed as per cent of the original resistance.

[8] Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxi, 585.

[9] This agrees closely with the temperature coefficient as determined in a previous investigation. *Cf.* Osterhout, W. J. V., *Biochem. Z.*, 1914, lxvii, 272.

[10] This procedure may displace the points on the curve so that where several curves are averaged it may be necessary to employ interpolation in order to average points on the same ordinate. In many cases curves were obtained by averaging the ordinates of death curves and recovery curves before multiplying by the factor.

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effect of this is to make the process appear to proceed at 15 instead of at 17°C. If the difference between the two curves is due wholly to difference in temperature this introduces no error, and if the difference is due in part to other factors, the error, if any, is less than the usual experimental error.

The advantages of this procedure are that we can employ for our calculations the constants already obtained for the standard curve and also compare the theoretical curves which start from the same points. This procedure has therefore been followed throughout and the corrected results (*i.e.* the figures multiplied by a suitable factor) are employed in the following description.

When the tissue was replaced in sea water the resistance began to rise. At the end of 10 minutes it had risen from 80.69 to 92.57 per cent.[11] Since, however, the abscissæ of the death curve have been multiplied by 1.06 the same thing must be done for the recovery curve and in place of 10 minutes we must put 10.6 minutes. Proceeding in this manner we obtain the recovery curve which is labeled 15.9 in Fig. 1.

In order to calculate the course of the recovery curve we must consider the reactions which determine the amount of electrical resistance. When the tissue is placed in the solution of NaCl the reactions which occur are: (1) $A \rightarrow M \rightarrow B$; (2) $R \rightarrow S \rightarrow T$; and (3) $N \rightarrow O \rightarrow P$. Let us first consider the reactions $A \rightarrow M \rightarrow B$. The value of A in sea water is taken as 2,700 and that of M as 90. As explained in a former paper[1] the value of A will diminish during exposure to NaCl according to the formula

$$A\, e^{-K_A T} \qquad (2)$$

Since $K_A = 0.018$ (see Table I) the value of A after 15.9 minutes in sea water is

$$2{,}700\, e^{-(0.018)\ 15.9} = 2{,}027.96$$

The value of M at the end of 15.9 minutes is the observed resistance 80.69 less 10 (since the base line of the curve is not 0 but 10).

[11] In earlier experiments it was found that complete recovery was possible after the resistance had fallen to about 80 per cent. This was not the case in the present series; the difference may be due to differences in material or in technique. *Cf.* Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 242.

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On replacing the tissue in sea water, therefore, we start with $M = 70.69$ and $A = 2{,}027.96$, but this value of A is at once augmented by the conversion of S into A. In order to find the amount of this augmentation we must know the value of S.

During exposure to NaCl the reaction $R \rightarrow S \rightarrow T$ occurs. The value of S may be easily calculated by employing formula (1) and substituting the appropriate constants. We thus obtain

$$S = R\left(\frac{K_R}{K_S - K_R}\right)\left(e^{-K_R T} - e^{-K_S T}\right) + S_0\, e^{-K_S T} \qquad (3)$$

in which S_0 denotes the value of S at the start of the reaction. The value of R in sea water is taken as 1,041.77 and that of S as 2.7. In the solution of NaCl the values of K_R (the velocity constant of the reaction $R \rightarrow S$) and K_S (the velocity constant of the reaction $S \rightarrow T$) are taken as 0.04998 and 0.02856 respectively (see Table I). Hence the value[12] of S at the end of 15.9 minutes is 447.26. When the tissue is replaced in sea water S is rapidly converted into A so that the total value of the latter becomes $447.26 + 2{,}027.96 = 2{,}475.22$.

On replacing the tissue in sea water $A = 2{,}475.22$ and $M = 70.69$. The resistance after any given time T in sea water is obtained by modifying formula (1) which becomes

$$\text{Resistance} = 2{,}475.22\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 70.69\left(e^{-K_M T}\right) \qquad (4)$$

The velocity constants K_A and K_M have the normal values in sea water, 0.0036 and 0.1080 respectively. Hence the resistance at the end of 10.6 minutes is 87.44.

We must likewise remember that on replacing the tissue in sea water the reactions $O \rightarrow S \rightarrow A$ recommence and produce a certain amount of A; this breaks down to form M, which in turn decomposes. The resulting amount of M may be easily calculated. It will be recalled that in sea water all processes are so adjusted that the amount of M remains constant; it is evident that if the reactions $O \rightarrow S \rightarrow A$ were suddenly to stop, allowing $A \rightarrow M \rightarrow B$ to continue, the amount of M would diminish. At the start the total resistance is 100. If O

[12] In general the greater the rise in recovery the greater the value of S, while the greater the fall the less the value of S.

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should stop producing this would diminish and we may call the loss of resistance L. Now if O were producing normally it would just replace this loss, so as to keep the resistance constant at 100: hence the amount produced from O in any given time will be equal to the loss L which would occur in that time if O were to stop producing.

When tissue is exposed to a solution of NaCl, O diminishes according to the scheme $N \rightarrow O \rightarrow P$. Assuming that at the start $N = 89.1$ and $O = 90$ we find[13] that the value of O after any given time (T) of exposure to a solution of NaCl may be obtained by changing the constants in formula (1) thus:

$$O = 89.1\left(\frac{K_N}{K_O - K_N}\right)\left(e^{-K_N T} - e^{-K_O T}\right) - 90\,e^{-K_O T} + 10 \qquad (5)$$

in which K_N (the velocity constant of the reaction $N \rightarrow O$) and K_O (the velocity constant of the reaction $O \rightarrow P$) have the values 0.03 and 0.0297 respectively (see Table I).

We find by this formula that at the end of an exposure of 15.9 minutes the value of O is 92.57; hence it can produce only $(92.57 - 10) \div (100 - 10) = 0.917$ as much of M in any given time as it could produce if it were intact.[14] The amount it could produce, if intact, during recovery in sea water is easily found by subtracting from 100 the resistance obtained by means of formula (1), when $K_A = 0.0036$ and $K_M = 0.1080$ (these are the normal values in sea water). Using these values we find that at the end of 10.6 minutes the amount of resistance, as given by formula (1), would be 98.55. Hence the loss during that time would be $100 - 98.55 = 1.45$, which is the amount

[13] This value of O is assumed merely for convenience in calculation without reference to other assumed values. Its real value must be much greater than that of A but it is not necessary to assign any definite real value to it, since the only point of interest is to determine what per cent of O remains after any given time of exposure to sea water. It is assumed that in sea water any change in the amount of O is so small as to be negligible. This might be due to the fact that O is present in large amount and decomposes slowly or to the fact that it is formed as rapidly as it decomposes (by the reactions $N \rightarrow O \rightarrow P$).

[14] In other words, if S, T, and A were completely removed, O could raise the level of M to $100 - 10 = 90$ in the course of time. But if, for example, half of O is lost the remainder can raise the level of M to only one-half its former value; *i.e.*, to $45 + 10 = 55$.

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O could produce in 10.6 minutes if intact: but as O has diminished to 0.917 times its original value it can produce in 10.6 minutes only (1.45) (0.917) = 1.33.

By adding this value to that obtained by formula (4) we find the resistance after 10.6 minutes in sea water to be 87.44 + 1.33 = 88.77.

In the same manner we may find the resistance at any given time after replacement in sea water. A series of values so obtained is given in Table II. It will be seen that they are in good agreement with the experimental values. The calculated and observed values are also plotted in Fig. 1, in which the abscissæ represent the time in the solution of NaCl plus the time of recovery in sea water (in the case just discussed this would amount to 15.9 + 10.6 = 26.5 minutes).

TABLE II.

Recovery in Sea Water after Exposure of 15.9 Minutes to 0.52 M NaCl.

Total time = time in sea water + 15.9.	Time in sea water.	Electrical resistance.	
		Observed.	Calculated.
min.	*min.*	*per cent*	*per cent*
26.5	10.6	89.10	88.77
37.1	21.2	93.21	91.34
58.3	42.4	93.99	92.43
84.8	68.9	94.48	92.52
121.9	106.0	94.20	92.55
164.3	148.4	93.81	92.55
545.9	530.0	94.00	92.57
863.9	848.0	93.87	92.57

Proceeding in this manner with different times of exposure we obtain the series of recovery curves shown in Fig. 1. The number attached to each curve denotes the time of exposure to the solution of NaCl. The observed results are plotted as dotted lines, the calculated values as unbroken lines.

It will be seen that the agreement is satisfactory throughout. In general the greater the number of experiments which were averaged to obtain the result the nearer it approached to the calculated curve.

Let us now consider the behavior of tissues transferred from a solution of 0.278 M $CaCl_2$ (which has the conductivity of sea water) to sea water. In such a solution the resistance rises and then falls. If

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tissue is allowed to remain in the solution for a short time and is then replaced in sea water the resistance falls rapidly, as shown in Fig. 2. This fall of resistance may be regarded as analogous to the rise of resistance which occurs in the experiments with NaCl and the term recovery may be used in both cases. It is evident from the figure that, as the exposure to the solution of $CaCl_2$ lengthens, the level

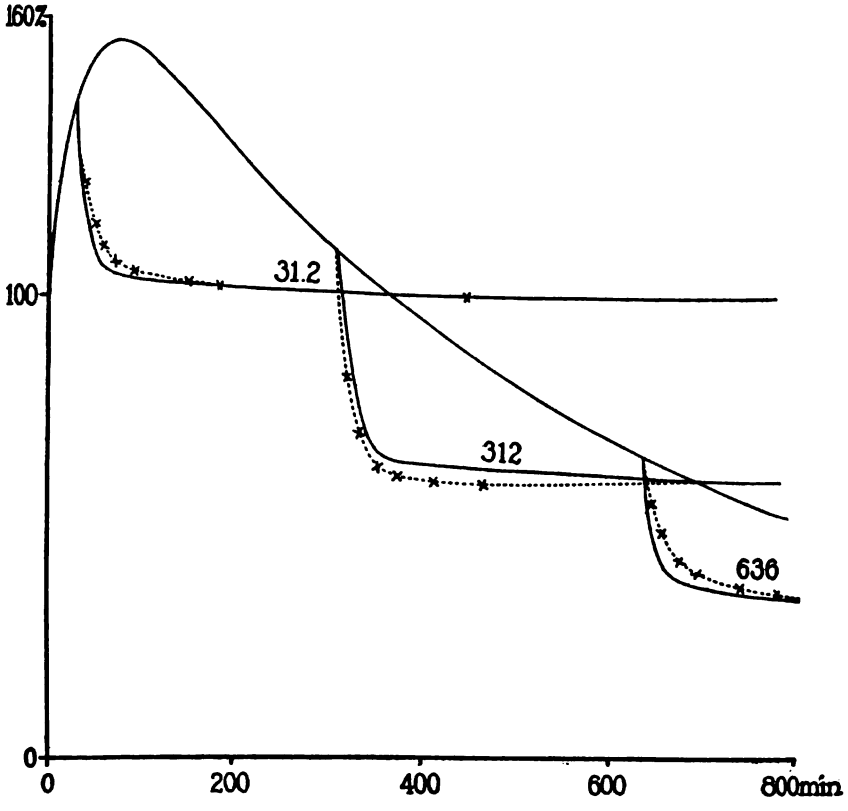

FIG. 2. Curves showing the rise and fall of electrical resistance in *Laminaria agardhii* in 0.278 M $CaCl_2$ (single curve which rises and falls) and recovery in sea water (descending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the solution of $CaCl_2$.

In the recovery curves the experimental results are shown by the dotted lines, the calculated results by the unbroken lines.

The observed points represent the average of eight or more experiments. Probable error of the mean less than 10 per cent of the mean.

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which is reached as the result of recovery gets lower. This is precisely what happens in the experiments with NaCl. It would therefore appear as though the same mechanism of recovery were involved. If this is so the same method of calculation should enable us to predict recovery in both cases. This is found to be true. Using the same formulas which have already been employed in the experiments with NaCl we are able to predict the course of the curves obtained in experiments with $CaCl_2$. This is rather striking in view of the fact that the two sets of curves differ so fundamentally in appearance.

In calculating the curves for $CaCl_2$ the constants given in Table I are employed. The results are shown as unbroken lines in Fig. 2 (the dotted lines show the experimental results). It is evident that the agreement is very satisfactory.

Some assistance in picturing the reactions which occur during exposure is afforded by Fig. 3, which shows the curve of O in NaCl (unbroken line) and in $CaCl_2$ (dotted line). These curves are plotted from the calculated values; the observed values are shown as points; it will be observed that they lie fairly close to the calculated curve. The figure also shows the calculated values of S: in this case no observed values are given because such values cannot be very precisely determined. This is owing to the fact that the value of S affects only the speed of recovery (not the final level attained) and as the speed is variable the only satisfactory procedure is to assume such values of K_R and K_S as cause the closest approximation to the observed speed of recovery. When these values have been found the value of S can readily be calculated. The results of these calculations are plotted in Fig. 3.

In this figure the ordinates give the values of O: these must be multiplied by 6.75 to obtain the values of S. In all curves the value of S at the start is 2.7 (the value of S in sea water[15]); this appears on the ordinate in the figure as $2.7 \div 6.75 = 0.4$. The curves rise to a maximum and then fall to zero. The curves for O start at 100 and fall to 10 (since the base line is taken as 10, just as in the curve of M).

[15] The normal value of S in sea water is taken as 2.7 which is exceedingly small as compared with the amount of O. The amount of S which is produced from O in each unit of time is relatively large but S is so rapidly transferred into A that its amount in sea water never becomes greater than 2.7.

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It will be observed that the rate of recovery is approximately the same in all cases; this applies to the experiments with $CaCl_2$ as well as with those in NaCl. In general it may be said that it usually requires about 60 minutes for the curve to complete nine-tenths of the total rise or fall which occurs in recovery.

With so large a number of constants it might seem possible to fit any sort of curve and hence the significance of the actual accomplish-

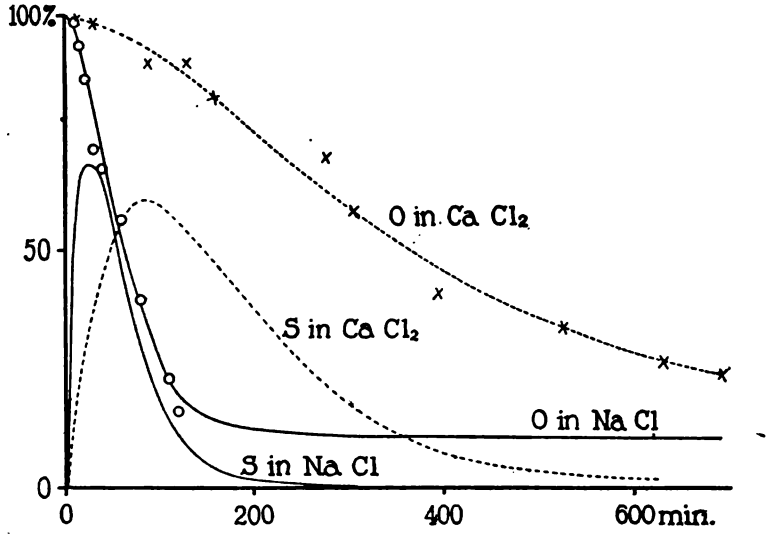

FIG. 3. Curves showing the values of *O* in NaCl (upper unbroken line) and in $CaCl_2$ (upper dotted line); also the values of *S* in NaCl (lower unbroken line) and in $CaCl_2$ (lower dotted line). The ordinates give the values of *O*; these must be multiplied by 6.75 to obtain the values of *S*.

The observed points represent the average of eight or more experiments; probable error of the mean less than 10 per cent of the mean.

ment might be lessened. This, however, is by no means the case. Moreover, the fixing of one or two constants affects the others in such a way as largely to determine the character of all the curves.

In the foregoing account many details are necessarily omitted, owing to lack of space. These, however, are not essential to the main purpose, which is to show how the process of injury and recovery may be analyzed and subjected to mathematical treatment. Starting

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with certain assumptions we have formulated equations by means of which we can predict the behavior of the tissue. If the predictions are fairly accurate it is natural to infer that the assumptions are in accordance with the facts. It is evident from an examination of the figures that the equations enable us to predict with considerable accuracy the behavior of tissues in solutions of NaCl and $CaCl_2$, as well as the recovery curves after any length of exposure to either of these solutions. But we must not lose sight of the fact that the predictive value of the equations does not depend on the validity of these assumptions and would in no way be impaired if they were to be given up. The equations have a permanent value which is independent of assumptions.

SUMMARY.

1. *Laminaria* exposed for a short period to 0.52 M NaCl loses a part of its electrical resistance but recovers it completely when replaced in sea water. When the period is lengthened recovery is incomplete. If the exposure is sufficiently prolonged no recovery occurs. (After exposure to 0.278 M $CaCl_2$ the resistance falls when the tissue is replaced in sea water.)

2. Equations are developed which enable us to predict the resistance of the tissue during exposure to NaCl or $CaCl_2$ as well as the recovery curves after any length of exposure to either of these solutions.

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LABYRINTH AND EQUILIBRIUM.

III. The Mechanism of the Static Functions of the Labyrinth.

By S. S. MAXWELL.

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The Static Functions of the Ampullæ.

In a preceding paper[1] I have shown that removal of the otolith organs from the labyrinth of the dogfish does not destroy the static functions; that, on the contrary, the compensatory changes of position of the eyes and fins occur when the animal so operated is tilted out of the normal or primary position; and that if the animal is held in the abnormal position permanently the compensatory positions of the eyes and fins are also retained permanently. I have also pointed out that the stimuli for the maintenance of these compensatory positions do arise from the labyrinth; that they are not affected by the exclusion of other sensory stimuli; but that they disappear at once when the remaining parts of the labyrinth are destroyed. I also proved that rotations around the body axes excite the dynamic responses through changes of tension on the ampullæ due to the inertia of the vestibular contents, and not through the production of currents in the semicircular canals. I wish now to show that the same mechanism which excites the ampullæ to the exercise of their dynamic functions serves also to produce their static effects.

While an animal is undergoing rotation around a body axis there is brought about through inertia a displacement of the contents of the vestibule, and this displacement and the consequent change of tension acts as a stimulus. If on cessation of the movement of rotation the

[1] Maxwell, S. S., Labyrinth and equilibrium. I. A comparison of the effect of removal of the otolith organs and of the semicircular canals, *J. Gen. Physiol.*, 1919–20, ii, 123.

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contents of the vestibule returned at once to their original position the stimulus would cease and the eyes would return to the primary position. If, however, the new position of the vestibular structures continued to exist after cessation of the movement the tension differences would also continue and the resulting stimuli would give rise to sustained forced position of the eyes; *i.e.*, to the static effect. The latter condition could exist in case the specific gravity of the utricular tissues is greater than that of the lymph. This I have found to be the case.

In most selachians the lymph of the vestibule is in free communication with the exterior sea water through the ductus endolymphaticus. It is reasonable therefore to expect that the density of the lymph would be practically equal to that of sea water. On this assumption I determined the relative weights of the membranous labyrinth and sea water by dropping small bits of utricle, ampullæ, and semicircular canals into a tall jar of sea water and saw that they all sank to the bottom. In order to be more certain, however, I succeeded in getting a sufficient amount of lymph from the ears of several fish killed at one time and dropped bits of the membranous labyrinth into it, with the result that they sank just as in sea water.

Since the membranous labyrinth and the lymph differ in specific gravity it is evident that when the membrane is displaced to a relatively lower position its weight will have the tendency to prevent its return to the original position in the cavity as long as the new body position is retained. I believe that this difference in weight, then, is the cause of the continued forced position in the absence of the otolith.

It has been frequently stated that the stimulation of an ampulla gives rise only to a momentary movement, not to a sustained forced position, and that therefore its function can be only dynamic and not static. I have found the contrary to be very definitely true. Sustained mechanical stimulation of an ampulla, even the ampulla of a horizontal canal, causes a sustained forced position of the two eyes; namely, a conjugate deviation to the side opposite to the stimulated ampulla. It is self-evident that in the ordinary functioning of the horizontal ampulla, when the rotation to which it responds is in a horizontal plane, no changed relation to gravity can occur and hence the reaction to rotation cannot continue after the rotation has ceased.

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That the horizontal ampulla reacts to its normal stimulus by a response then is due only to its space relations, and not to a different kind of physiological function. Of course my experiments have demonstrated the ability of the other ampullæ to produce sustained static effects.

The Static Functions of the Otolith Organ.

In the dogfish and related forms it is comparatively easy to remove all the ampullæ and to see that in their absence both static and dynamic functions are retained by the remaining parts of the labyrinth; that is, compensatory movements and compensatory positions of the eyes and fins still occur in response to rotations in all planes except the horizontal. The removal of the large otolith of the sacculus has no effect whatever on these reactions; but if now, in addition, the small otolith of the recessus utriculi is removed the compensatory movements are at once abolished.

The original suggestion of Breuer that the otolith organs constitute the apparatus for the static functions of the labyrinth has been widely accepted. It has also been generally believed that the pressure of the otolith due to its weight is the stimulus which gives rise to the static function. If, for example, the animal is rotated to the right around its longitudinal axis and held in this new position, the pressure of the otoliths is shifted to the right and presumably the epithelium on the right side of the macula is now subjected to a relatively greater pressure, or even a previously unstimulated portion is now brought under pressure. In a previous paper[2] I was inclined to accept a similar explanation of the dynamic functioning of the otolith. In the light of new experiments to be reported below I am led to modify this view. I had, however, long ago given proof that in the compensatory movements of the horned toad (*Phrynosoma*) the exciting cause is not pressure, *per se*, but the torsion effect due to rotation.[3]

[2] Maxwell, S. S., Labyrinth and equilibrium. II. The mechanism of the dynamic functions of the labyrinth, *J. Gen. Physiol.*, 1919–20, ii, 349.

[3] Maxwell, S. S., On the exciting cause of compensatory movements, *Am. J. Physiol.*, 1911–12, xxix, 367.

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Simple and plausible as Breuer's *a priori* conception appears no one has ever been able to confirm it by direct experiment. Lee,[4] for example, states that: "Stimulation of the otolithic parts of the ear has not been entirely satisfactory. The results were found exceedingly variable." Experiments by pressure on the otoliths or epithelium have usually been described as unconvincing or contradictory. As will be seen they are consistently contradictory to Breuer's views so commonly held.

I have now found that it is possible to stimulate mechanically the otolith organ of the recessus and to get results just as clear and consistent as those obtained from stimulation of the ampullæ. I quote the following record of an experiment.

"July 16, 1920. Large dogfish (*Galeus*).
Opened both ears and removed all six ampullæ.
Using a stiff bristle tipped with wax and the wax covered by a thin layer of absorbent cotton, applied pressure to various parts.
Right ear.
Pressed on right (lateral) side of otolith (of recessus utriculi); right eye depressed, left eye elevated.
Pressed on left side of otolith; left eye depressed, right eye elevated.
Repeated several times with uniform results. Otolith soon disintegrated: no more response.
Left ear.
Pressed on left side of otolith; left eye depressed, right eye elevated.
Pressed on right side (median) of otolith; right eye down, left eye up.
Repeated several times with same result."

Experiments made in this way gave fairly constant results, but it was not possible to repeat the observation many times without injury to the otolith organ. A new and very simple method was later found which permitted repetition of the stimulation many times before serious damage was done to the otolith and which gave absolutely constant results. The experiment is performed as follows:

A small mass of absorbent cotton is formed into a tiny cushion about the size of the otolith of the recessus and is cautiously placed on top of that otolith. The cotton is then grasped with the points of a fine

[4] Lee, F. S., A study of the sense of equilibrium in fishes. I, *J. Physiol.*, 1894, xv, 311.

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forceps and gently moved to the right or left, forward or backward at will. I quote again from my notes:

"July 23, 1920. Large shovel-nosed ray (*Rhinobatus*).
Removed ampullæ from left ear.
Exposed small otolith (of recessus utriculi) and placed on it the pellet of cotton.
Movement of pellet to left caused depression of left eye and elevation of right eye.
Movement of pellet to right caused depression of right eye and elevation of left eye.
Movement of pellet forward caused both eyes to roll forward on their axes (anterior pole of each eye depressed and posterior pole elevated).
Movement of pellet backward caused both eyes to roll backward on their axes.
When pellet was moved to one side eyes moved in same sense.
When pellet was held to any side, the eye position was retained.
Removed the three ampullæ of the right ear.
Repeated the experiment on the right ear with exactly the same results.
Repeated a score or more of times with no noticeable diminution of the response.
Holding the pellet to any side held the eyes in the corresponding position."

I have repeated these experiments on dogfish, leopard sharks, and rays. The experiments on the ray (*Rhinobatus*) were particularly striking. This fish is broad and flat and usually remains on or near the bottom of the water. It is not apparently used to much turning over or tilting of the head up or down. Taken out of the water, or rotated in the water it does not show any of the compensatory movements in so marked a degree as does the dogfish. When, however, the stimulation was applied to the recessus as described above, the eye movements were extraordinarily vigorous, much more so than in response to rotation of the body of the uninjured animal. The eyes rolled right or left, forward or backward as if on actual mechanical axes manipulated by cords.

It will be seen from the above that pressure on the *right* side of the otolith of either ear produces the same eye movement which results as the compensatory motion to rotation of the body to the *left* around the longitudinal body axis; and that pressure on the *anterior* side of the otolith gives the same effect as tilting the head *upward*. In each case the response is precisely opposite to that which would be expected if the stimulation were produced by the pressure due to the weight of the otolith; for when the body is tilted to the right the weight of the

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otolith must be shifted to the right, but the reaction of this rotation is elevation of the right eye and depression of the left eye. When pressure is applied directly to the right side of the otolith as in the experiments above described the opposite result is obtained; namely, depression of the right eye and elevation of the left. It must be then that the stimulation does not result from the direct effect of the pressure but from the shifting of the otolith; a displacement to the left is brought about by pressing on its right side under the conditions of the experiment, and a similar displacement to the left results from tilting the animal to the left. In other words, it is the *displacement* of the otolith, and not the pressure due to the weight of the otolith, which is the actual stimulus and it is the *direction* of the displacement which determines the direction of the compensatory movement in response to the stimulus.

The experiments described above show that the stimulus arising from the position of the otolith is not due to the pressure as such but to the relative tensions, and is in this particular exactly similar to what I had already found for the ampullæ. The stimulus which causes the forced position (static function) is, like the stimulus which causes the compensatory movement (dynamic function), due to relative differences of tension in the organ, rather than to localized stimulation of special portions. There is therefore no evidence for a specific difference in the mode of action of the various parts of an ampulla or a macula. The relative tensions appear to determine the proportionate nerve excitation for the associated muscle groups in a manner analogous perhaps to the effects of various degrees of tension in the lungs on the vagus endings. I have made no attempt to say just where these differences of tension take effect upon the nerve endings, nor to say what part, if any, the hair cells have in the process. The arrangement of the hair cells would seem admirable for the transmission of the effects of movement or pressure to the nerve endings, but I do not at present see how the matter can be subjected to the test of experiment.

A part of the experiments on which this paper is based were performed at the Scripps Institution for Biological Research. I take great pleasure in acknowledging my obligation to the Director, Professor W. E. Ritter, for his courtesy in placing the facilities of the Institution at my disposal.

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THE ACTION OF INHIBITORY NERVES ON CARBON DIOXIDE PRODUCTION IN THE HEART GANGLION OF LIMULUS.

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While stimulation of nerves generally results in increased activity, the vagus has the opposite effect of diminishing the activity of the heart. The mechanism of this inhibition has not yet been explained. The author's recent experiments on the heart ganglion of *Limulus polyphemus*[1] have shown that stimulation of the inhibitory nerves of this heart results in a decrease in the production of CO_2 in the ganglion, while direct stimulation of the ganglion by electrical or mechanical or chemical means has the opposite result, namely an increase in CO_2 production in the heart ganglion, as will be shown in a later paper. This leaves no doubt that the action of the inhibitory nerves upon automatic ganglia is due to a decrease in those chemical processes in the ganglion which result in the formation of CO_2.

Methods.

The rate of CO_2 production was determined by the method previously described by the author.[2] Briefly, the ganglion, with its inhibitory nerve is dissected from the heart and immersed in 3 cc. of a

[1] The special suitability of the heart ganglion of *Limulus* arises from the fact that the heart may be isolated from the animal but beats for a day or more, likewise the ganglion may be dissected free from the heart and if connected with one segment alone will continue to deliver its impulses to the muscle for hours in sea water or in the solution used in this investigation. We are thus able to make our tests without concern about questions of changes of blood pressure, of vasomotor action, of chemical composition of the medium, or of the secondary action of other nerve supply which so complicate our experiments on the vertebrate nerve centers.

[2] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 41, 49.

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physiological saline solution consisting of 100 cc. of M/2 NaCl plus 2 cc. of M/2 $CaCl_2$ and containing phenolsulfonephthalein to indicate the hydrogen ion concentration.

We note the time required to produce the color change which indicates a change in the hydrogen ion concentration from the initial value pH 7.8 to 7.4. This gives a normal standard of the rate of development of CO_2 which can be compared with the rate at which the ganglion produces the same change while the inhibitory nerves are being stimulated.

Owing to the many failures to secure preparations of the ganglion with the inhibitory nerve uninjured and functioning, the following technique was adopted. Upon opening the dorsal carapace and pericardium the inhibitory nerves were identified by faradic stimulation; they were then secured distally with a fine silk ligature. The pericardium was removed from the whole heart and the cardiac ganglion, from its posterior end to the middle of the second segment, was dissected from the heart. This left functional connection of the ganglion with the first muscular segment which therefore continued to beat. The ganglion, free from all extraneous tissue, was now looped over tiny hooks on a slender glass rod which was cemented into the stopper of the indicator tube and the inhibitory nerves connected with it were laid across fine platinum wire electrodes passing through and cemented into the stopper. The nerves were again stimulated to determine whether they still inhibited the ganglion and stopped the beats of the first muscular segment. If the dissection has been successful it is only necessary to snip the nerve cord anterior to the point at which the inhibitory nerves enter to obtain the desired preparation of the ganglion mounted and ready for introduction into the tubes containing the solution with the indicator (phenolsulfonephthalein). We now determine the rate of the desired color change.[3]

[3] In a simpler preparation the ganglion alone was used and stimulation applied to its anterior end. This usually produces inhibition but stronger faradization may stimulate, so that the analysis of the results of the respiratory changes was sometimes problematical, therefore we have confined our published results to those obtained by the method described in the text.

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Results.

The results of several typical experiments are summarized in Table I. The normal values for the time required to produce the change in pH are obtained before and after stimulating the inhibitory nerves and compared with the corresponding time while the stimulation is maintained. Differences in the time normally required by the different preparations to produce the change are due in part to differences in the size of the ganglia and in part to inherent differences in the

TABLE I.

Rate of CO_2 Production, before, during, and after Stimulation of Inhibitory Nerves.

	CO_2 production expressed in time required to produce color change.				
Experiment No.	1	2	3	4	5
	sec.	*sec.*	*sec.*	*sec.*	*sec.*
Normally. *a*	98	262	130	179	72
Normally. *b*	113	250	130	184	—
Normally. *c*	102	—	—	—	—
During inhibition	258	848	268	289	154
	—	—	242	—	—
After inhibition. *a*	197	546	154	201	78
After inhibition. *b*	124	314	—	—	75
After inhibition. *c*	120	286	126	180	—

intensity of the processes of CO_2 production. The results obtained by these experiments deal essentially with relative rates of CO_2 production, no attempt having been made to determine absolute values for the amount of CO_2 produced.

Analysis of the experiments leaves no doubt that inhibition of the automatic cardiac ganglion is a process involving a well marked depression of the CO_2 production in the ganglia (20 to 60 per cent of normal). The fact that the carbon dioxide production is only retarded and not completely suppressed is probably due to incomplete inhibition or to escape from inhibition toward the end of the period during which the determination is being made, since it has always been

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noted that the rate at which the color change progressed was more rapid toward the end of a determination when the ganglion was presumably gradually escaping from inhibition.

In preparations showing a low initial rate of CO_2 production it is easy to depress this rate by inhibition for a much longer time than with vigorous preparations. In one experiment (not tabulated) no color change was noted at the end of 30 minutes of inhibitory nerve stimulation, although subsequently the normal rate of 5 minutes was restored. These observations are in complete harmony with what we know about inhibition of the contraction rate in this form.[4]

TABLE II.

Effect of Strength of Inhibiting Stimuli on Time For CO_2 Production.

Experiment No.	Normal (average).	Weak inhibition. Harvard coil. (13 to 10 cm.)	Strong inhibition. Harvard coil. (7 to 4 cm.)
	sec.	*sec.*	*sec.*
1	131	197	276
2	201	237	309
3	167	216	388
4	259	402	734
5	172	327	498
6	78	112	286
7	118	196	243

Since strong stimuli applied to the inhibitory nerves of the heart produce a greater slowing of the rate of heart beat than weak stimuli, experiments were made to see whether they produce a similar effect upon the rate of CO_2 production in the ganglia. Some typical experiments are given in Table II.

The results leave no doubt that the stronger inhibiting stimulation, which is known to produce a slower heart beat, also causes a greater depression of the rate of the chemical processes in the ganglia which give rise to the formation of CO_2.

If we view the entire process of inhibition we may represent the experimental results graphically as has been done in Fig. 1 which is constructed for four of the experiments in Table I. The graph shows

[4] Carlson, A. J., *Am. J. Physiol.*, 1905, xiii, 217.

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that the drop in the rate of CO_2 production follows abruptly upon the incidence of inhibition but that the return to the normal after inhibition is a much more gradual process, indicating a persistence of the inhibitory state after the cessation of stimulation of the inhibitory nerves. The recovery to the normal rate of CO_2 production is slower the greater the degree of depression during inhibition.

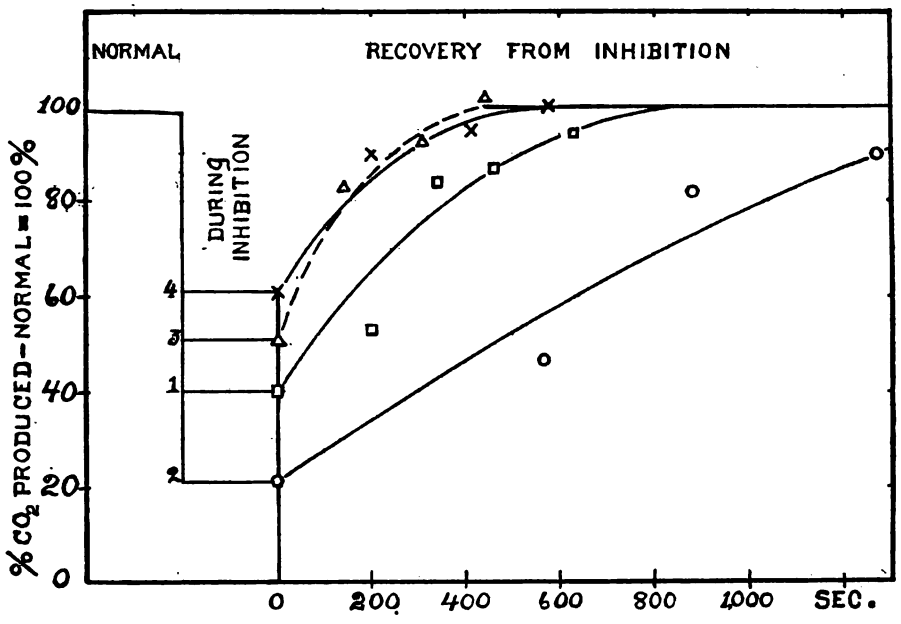

FIG. 1. Curves showing the percentage rate of CO_2 production compared with normal = 100 per cent. The numbers 1, 2, 3, and 4 correspond to the experiment numbers in Table I. The horizontal levels opposite these numbers show the average depression of CO_2 production during the period of inhibition which in each case continued for the time required to make the determination (*cf.* Table I). The readings for the curves of recovery are designated for each experiment by the character at the end of the inhibition period. Recovery time in seconds.

It is significant that the recovery process involves a return merely to the previous norm and that there is no subsequent increase in the rate of CO_2 production, no acceleration or after-augmentation, so that experimental evidence lends no support to the view that inhibition as exemplified in these nerve cells is accompanied by any con-

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structive or anabolic processes. The latter processes are typical of arrest,[5] but mere cessation of action, such as occurs in blocking of impulses, is not to be confused with true inhibition which must be conceived as synonymous with depression of the chemical processes concerned in CO_2 production in the nerve cells. When this conception of inhibition is applied to other types of cells as well as to nerve cells, it will do much to clear up conflicting and confusing ideas concerning the true nature of the inhibitory mechanism.

SUMMARY AND CONCLUSIONS.

It has been shown in this paper that stimulation of the inhibitory nerves of the neurogenic heart of *Limulus*, which correspond to the vagus nerves of the vertebrate heart, results in a marked diminution of CO_2 production in the heart ganglion, while stimulation of the ganglion, leading to increased activity of the heart, leads also to increased CO_2 production by the ganglion. This shows that inhibition of the automaticity of this ganglion by the action of its inhibitory nerves consists, not in a process of blocking, but in a diminution of those chemical reactions in the ganglion cells which give rise to the production of CO_2.

[5] *Arrest* may be quite independent of inhibition and due to secondary causes such as blocking or removal of stimulating impulses. This is exemplified upon stimulation of the vagus fibers to the turtle heart; the auricles are inhibited (true depression), the ventricles merely arrested, and the latter only show the after-augmentation.

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THE KINETICS OF INACTIVATION OF COMPLEMENT BY LIGHT.

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In order to determine the course of an ordinary chemical reaction it is only necessary to mix the reagents and then to withdraw samples for analysis at appropriate intervals. But in the study of light reactions the case is far different, because the dimensions and transparency of the radiated material exercise a preponderant influence on the rate of reaction at any given intensity of illumination.

Since a great part of the difficulty encountered in studying the course of complement photoinactivation is due to the peculiar requirements imposed by these facts, which are often inadequately considered in connection with physiological problems, we shall begin with a discussion of these requirements, and of the means adopted to meet them.

I.

In the first place, since the intensity of the light reaching each part of the solution determines the rate of reaction at that point it is essential that the average intensity of the light reaching each part of the reaction mixture should remain constant during that part of the course studied. This is a condition which can be met only by special arrangements: either by having a cylindrical reaction chamber parallel to a relatively long "line source" of light, and withdrawing samples at intervals; or by exposing successive samples for a series of different periods under otherwise identical conditions. The latter alternative was adopted in these experiments.

* The experiments upon which this paper is based were done by the writer as Research Fellow in the Harvard School of Tropical Medicine.

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In any case all parts of the radiated material must be exposed to essentially the same light intensity; otherwise the true order of the reaction cannot appear. In the case of serum, which is quite opaque to ultra-violet light, the part of the solution furthest from the light is shaded by the intervening serum. The opacity or absorption index of serum is so great that the back surface of a layer of complement diluted as much as practicable and only 0.2 mm. deep would still receive only 0.9 as much light (of the wave-lengths affecting complement) as the front surface. When the radiated material is a solution it can be vigorously stirred so that each portion is exposed to high and low light intensity alternately. In the case of a serum solution stirring is the simplest method of exposing all the solution to the same *average* light intensity.

Even with the two precautions indicated above, it is possible to determine the kinetics of a photochemical process only under certain restricted conditions which are not under the control of the experimenter, but inherent in the solution investigated. The existence of these conditions must be established by appropriate experiments before conclusions can be drawn as to the nature of the photochemical process itself. There are two points to be established: the absence of conditions causing progressive changes in the amount of effective light; and the negligibility of diffusion as a limiting factor in controlling the rate of reaction.

II.

If a reaction appears to be monomolecular, as complement photoinactivation does, it is always possible that this is the case simply because the reaction occurs with great velocity at some particular region, such as for example the walls of the containing vessel. The reaction will then appear to be monomolecular solely because the velocity of the reaction is limited by the amount of the substance arriving in the reaction field by diffusion, and, as the reaction proceeds, the rate of this diffusion decreases exponentially in accordance with Fick's Law.[1]

[1] Fick, A., *Ann. Phys. Chem.*, 1855, xciv, 59.

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But diffusion is a process which is accelerated to a characteristic degree by an increase of temperature, and its presence as a limiting factor can be detected by conducting the reaction at different temperatures and noting how much its velocity is affected. The temperature coefficient, Q_{10}, for diffusion is 1.22 to 1.28, for "dark" chemical reactions usually 2.0 to 3.0 or above, and for light reactions 1.0 to 1.1. Some light reactions, notably those in which light does no work but acts merely as a catalyst, are exceptional in that they have higher coefficients.

Experiments were carried out early in the course of these studies to determine the temperature coefficient of the process of photoinactivation. A sample of complement was diluted to 2 per cent and divided into portions of 20 cc. each. These were exposed for different lengths of time at each of several different temperatures (0, 10, 20, 30, or 40°C.). The exposure was made in the manner previously described[2] except that the complement was maintained at the temperatures above 0° by partial immersion of the container in a water bath which was kept within 1° of the desired temperature. The calculation of reaction velocities is based on the assumption that the reaction is monomolecular. It will be apparent from data given further on in this paper that this is the most nearly accurate simple assumption which is possible (Table I).[3]

[2] Brooks, S. C., *J. Med. Research*, 1918, xxxviii, 345.

[3] The samples exposed at 30 and 40° are injured by heat as well as by light. The efficiencies given in Table I have been corrected as follows: the average rate of heat inactivation was calculated from the injury suffered by duplicate samples kept in the same water bath with each radiated sample for the corresponding length of time. Both photoinactivation (Section V of this paper), and thermoinactivation (Madsen, T., and Watabiki, T., *Overs. kong. danske Videnskab. Selskabs Forhandl.*, 1915, 125) follow the course of a monomolecular reaction and their observed velocities can be expressed as the velocity constant, k, of the monomolecular reaction isotherm, $k = \frac{1}{t} \log \frac{a}{a-x}$. By subtracting from k_O, the observed velocity when light and heat are both acting, the value k_H, observed when only heat is acting, we arrive at a value, k_L, which we may consider to be the component of the velocity due to the light alone. k_H was found to be 0.010 at 40 and 0.005 at 30°C. These values were subtracted from the k_O for each exposure, and from these values of k_L there were obtained (by the use of the reaction isotherm) corrected values of the efficiencies to be expected if light alone were to

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In Fig. 1 the results of this experiment are graphically presented with the efficiencies of the different portions of complement plotted as ordinates against their respective periods of exposure as abscissæ. The smoothed curves drawn between the points for any one temperature allow us by interpolation to determine the exposure necessary at that temperature to reduce the efficiency of complement by any given amount. The rate of photoinactivation may be taken as inversely proportional to the time required to reduce the hemolytic power of complement to any selected per cent of efficiency. Calculation from such interpolated values of the average rate of inactivation

TABLE I.

The Efficiency of Samples of Complement Radiated at Different Temperatures for Different Intervals.

0°C.		10°C.		20°C.		30°C.		40°C.	
Exposure.	Efficiency.	Exposure.	Efficiency.	Exposure.	Efficiency.	Exposure.	Efficiency.	Exposure.	Efficiency.
min.	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*
4.0	65.0	4.0	—	4.0	63.8	3.5	64.7	2.5	62.4
7.0	41.8	7.0	52.5	7.0	53.2	5.0	56.3	3.5	62.6
10.0	42.8	10.0	41.8	10.0	38.0	8.0	45.3	5.0	47.3
14.0	27.8	14.0	26.2	14.0	25.9	12.0	29.7	8.0	39.8
21.0	12.0	21.0	11.0	21.0	—	17.0	15.3	12.0	28.4

to 60, 40, and 20 per cent efficiency shows that if the velocity at 0°C. is taken as 1.00 the velocities at 10, 20, 30, and 40°C. are 1.02, 1.09, 1.25, and 1.47, respectively. Hence the temperature coefficients for the four intervals studied are Q_{10} = 1.02, 1.07, 1.14, and 1.18. These values are probably accurate to the second significant figure only, so that although there appears to be a progressive increase in

act upon the complement. These values are given in Table I. The heat effect is negligible at temperatures of 20°C. or below, so that $k_O = k_L$ and no correction need be introduced at these temperatures.

These experiments were done previous to the perfection of the method for complement titration used for the remainder of the work described in this paper, and, as might be expected, there are occasionally large inaccuracies. These are obviously to be neglected in certain instances such as the 7 minute exposure at 0°C.

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their value as higher temperatures are approached it would be premature to assume that such an increase would always be found. The mean value of Q_{10} is 1.10 and may be considered to be typical of the reaction. It is exactly that found in a preliminary experiment in which the rates of photoinactivation at 2 and $\frac{1}{2}$ at 38°C. were determined.

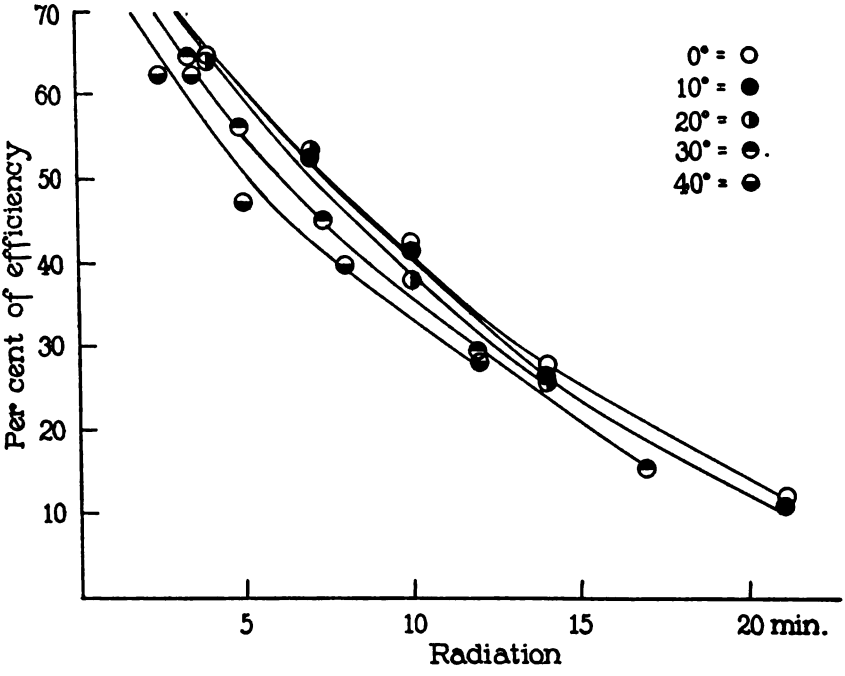

FIG. 1. Courses of the process of photoinactivation of complement at 0, 10, 20, 30, and 40°C. The ordinates represent efficiency in per cent of that of unradiated complement subjected to the same temperature conditions; and the abscissæ represent the time of exposure to light in minutes.

This value of the temperature coefficient for the process of photoinactivation is of particular significance because it is such as to allow us to neglect diffusion as a factor of any importance in determining the course of the reaction. For if diffusion were a limiting factor in the reaction the temperature coefficient of photoinactivation should be that of diffusion; *i.e.*, $Q_{10} = 1.28$. Instead of this it is 1.1 which is about the value which is to be expected in a photochemical reaction.

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III.

But the progress of the light reaction is in turn affected by the amount of light absorbed, and by the photochemical efficiency of the absorbed light. We may assume that the latter remains constant; that is, that the same amount of light energy is needed at any stage of the process to make a single molecule react. It is conceivable that this might not be the case; but the very fact that the essential reaction involved in the photoinactivation of complement seems to follow the laws of a simple chemical reaction shows that such a possibility is remote.

It was to be feared, however, that the transparency of a sample of serum would change during the course of photoinactivation. This would have made it difficult or impossible to interpret the experiments. If the transparency does remain constant it may do so for any of several reasons. On the one hand the photolabile substance alone may absorb the light (photoproducts not absorbing) and be present in sufficient concentration and depth to absorb all the light. In this case the true order of the reaction is greater than the apparent order by the number of photosensitive molecules participating. Thus a bimolecular reaction appears monomolecular. It is obvious that as such a reaction progresses and the photosensitive substance disappears, the solution will ultimately transmit some light and the order of the reaction can then no longer be calculated. On the other hand the transparency may remain constant because a negligible portion of the absorption is due to the photosensitive substance or because the products of the light reaction have the same absorption coefficient as the reacting substances. In either of these cases the intensity of the light impinging upon the photosensitive molecules is constant, the amount of light absorbed may be assumed to vary as the concentration of absorbing molecules, *i.e.* Beer's Law,[4] and, if the solution is well stirred, the apparent order will be the same as the true order.

It can be shown that a layer of complement of the depth and concentration used in determining the course of photoinactivation absorbs practically all the effective light, and that the amount absorbed remains constant during the reaction; *i.e.*, that the photosensitive

[4] Beer, A., *Ann. Phys. Chem.*, 1852, lxxxvi, 78.

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substance is constantly exposed to the same light intensity. Therefore if samples of complement are exposed to the light successively for varying periods, and well stirred during the exposure, the apparent order of the reaction will be the true order.

The transparency of complement was studied in the following way. A 5 per cent solution of fresh guinea pig serum in a physiologically balanced solution[5] was made up immediately after the serum was taken from the clot. Except during exposure to light the complement was kept at 0–1°C. The method of titrating the complement has been described in detail in a separate paper.[5] It consists essentially in allowing the complement to act on a suspension of specifically sensitized sheep erythrocytes suspended in the balanced solution previously mentioned, and noting the amount of each sample of complement required to produce a given amount of hemolysis in a given length of time. The relative efficiency of the various portions of complement was assumed to vary inversely as these amounts and was stated in per cent of the efficiency of an unradiated portion of the same complement.

It seemed desirable to determine approximately the absorption coefficient of solutions of complement of the concentration used; for from such observations it would be possible to determine what proportion of the incident effective light was absorbed in the apparatus used, and also, by analogy with the known absorption of serum, to determine the general region of the spectrum which was causing the photoinactivation.

To obtain such an approximate value for the absorption coefficient a 5 per cent solution of complement was placed in the inner of two concentric quartz test-tubes, and the balanced salt solution in the annular space between the two tubes; the space was 1.6 mm. wide. This set of tubes was then placed in a vertical position 9.5 cm. from a quartz mercury-vapor arc lamp and rotated on its long axis during an exposure of 3 minutes, which was exactly limited by the interposition of an opaque screen before and after radiation. Another portion of 5 per cent complement was then radiated in exactly the same manner except that the space between the two tubes (which

[5] Brooks, S. C., *J. Med. Research*, 1920, xli, 399.

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were always the same ones) was occupied by 5 per cent complement. The ratio of the velocities of photoinactivation in the inner tube in the two cases is the same as the ratio of the intensities of the light incident on the inner tube in the two cases, or in other words, since the salt solution absorbs a negligible amount of light, as the ratio of incident to emergent light when the complement is in a layer 1.6

TABLE II.

The Efficiencies in Per Cent and Velocities of Inactivation of Paired Samples of Complement, One Sample of Each Pair Being Radiated through a Layer of Complement and the Other through a Similar Layer of Balanced Salt Solution.

Sample.	Efficiency after exposure.	Velocity of inactivation.	Absorption coefficient.*
	per cent		
1*a*	87.4	0.020	74.8
1*b*	92.8	0.011	
2*a*	83.9	0.026	144.8
2*b*	94.9	0.008	
3*a*	91.5	0.0127	167.0
3*b*	96.4	0.0033	
4*a*	79.8	0.0327	54.1
4*b*	86.5	0.0212	
Average			110.2

* In the last column are given the absorption coefficients, μ, calculated from the formula $\mu = \frac{1}{dc} . ln \frac{I_0}{I}$; where d = thickness of the absorbing layer in centimeters, c = concentration of absorbing substance, and I_0 and I incident and emergent light intensities.

mm. deep. Four trials were made and the corresponding absorption coefficients calculated as shown in Table II. In calculating the velocities of reaction, as in the case of Table I, the reaction is supposed to follow a monomolecular course. The rather great divergence between the four observed values is probably explained by variations in the amount of pigment in the different samples of serum.

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The average of the four values of the absorption coefficient is 110.2 and may properly be considered to be of the true order of magnitude. This is about the absorption to be expected if light of a wave-length of about 2530 Ångström units were acting, while light of shorter wave-lengths is much more strongly absorbed.[6] The mercury arc emits little light of wave-lengths intermediate between that of a line at about 2530 Ångström units and that of about 2930 Ångström units, and light of wave-length greater than about 2900 Ångström units has little or no effect on complement.[7]

We may conclude that the principal effect of light on complement is due to ultra-violet light having a wave-length of about 2530 Ångström units; and that complement in layers of the depth used in the experiments on the course of photoinactivation absorbs a large part of the available light. For example, the most transparent sample, having an absorption coefficient of 54.1, would absorb nearly 95 per cent, the most opaque sample more than 99 per cent, of the incident light of the effective wave-length.

IV.

It was necessary, as has been pointed out in the preceding section, to determine whether or not there were changes in the transparency of complement during the progress of photoinactivation. To settle this point successive portions of complement were exposed for exactly equal lengths of time in the inner of the two concentric quartz tubes arranged as described above. In the space between the two tubes was placed a portion of the same solution of complement; this portion was left throughout the series of exposures. The total time of radiation of this sample varied in different experiments between 18 and 25½ minutes, and was long enough to reduce the efficiency of the complement to less than that attained in any of the experiments on the course of photoinactivation. Control experiments were conducted which were similar except that the outer space was filled with distilled water or with complement which had been completely inactivated

[6] Henri, V., Henri, Mme. V., and Wurmser, R., *Compt. rend. Soc. biol.*, 1912, lxxiii, 319.

[7] Bovie, W. T., *J. Med. Research*, 1918, xxxviii, 335.

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by long exposure to the light before the first sample was placed in the inner tube. If there were changes in transparency during photoinactivation successive samples should vary in their efficiency, and this variation should be greater than that displayed by successive samples in the control experiments. Examination of Table III will show that such is not the case and that therefore the transparency of the complement solutions remains constant during photoinactivation. In no case does the probable error of a single reading exceed

TABLE III.

The Variation in Efficiency of Portions of Complement Successively Exposed to Light in the Inner of Two Concentric Quartz Tubes for Equal Lengths of Time.

	Space between tubes filled with							
	Fresh complement.					Previously photoinactivated complement.		
Experiment No.	79	84	85	94	95	82	92	93
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Efficiency, in per cent, of successive portions.	84.7	96.7	94.0	95.8	85.0	86.7	90.9	95.2
	85.0	95.4	96.1	94.6	88.1	88.0	93.2	94.7
	86.2	97.8	96.3	95.2	86.0	89.1	93.8	94.2
	85.3	92.5	—	93.1	88.8	87.2	93.2	95.8
	85.1	95.5	—	89.9	85.9	87.1	91.5	95.9
	85.7	—	—	91.8	89.1	—	93.8	—
Mean efficiency.	85.3	95.6	95.5	93.8	87.5	87.6	92.7	95.2
Probable error of a single determination.	0.4	1.3	0.9	1.1	1.2	0.6	0.8	0.6
Mean probable error.	0.96					0.67		

that to be expected as a result of errors inherent in the method of titration, and although the mean calculated probable error is slightly less in the controls the difference cannot be considered significant.

We may sum up all these preliminary experiments with the statement that the apparent order of the reaction is the true order, because the effective light (probably that having a wave-length of 2536 Ångström units) is absorbed by complement in the same degree during all stages of photoinactivation. With the arrangement used

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in the experiments about to be described, more than 95 per cent of the incident light of the effective frequency is absorbed by the serum solution.

The time curves of complement photoinactivation which are given in the following section are therefore susceptible to analysis in terms of chemical kinetics; if the process follows the course of any simple chemical reaction it may be interpreted in terms of the number of kinds of molecules participating in the reaction.

V.

The course of complement photoinactivation was determined by radiating a number of portions of the same lot of complement for different periods of time, and plotting their efficiencies as ordinates against their respective periods of exposure as abscissæ. The curve connecting these points is assumed to represent the progressive change in efficiency which would occur in a single sample of complement. It was important that there should be no doubt about the fact that the amount of light action was characteristic of the particular serum used, and not simply an amount which might vary according to accidental differences between different exposures. To establish the fact that a given exposure would always cause the same amount of injury to a given complement solution three portions of complement were exposed for 10 minutes each, and then titrated. Their efficiencies were found to be 56.8, 54.4, and 54.7 per cent respectively; the mean efficiency was 55.3 per cent and the probable error of a single determination was 0.87 per cent which is 1.6 per cent of the mean value. This may be attributed entirely to errors in titration. Further examples of constant amounts of injury caused by short equal exposures may be found in Table III above.

Table IV and Fig. 2 present the course followed by the process of photoinactivation of eight different sera. They include all the dependable data obtained, a large number of the earlier experiments having to be discarded because of the difficulty of so arranging the titration as to include for each partially inactivated sample just those dilutions needed to yield a complete "titration curve." While it is probable that other types of inactivation curves might be encountered in the

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TABLE IV.

Efficiency of Portions of Eight Complement Solutions Radiated for Different Periods, and the Corresponding Values of the Term $k = \frac{1}{t} \log \frac{a}{a-x}$.

Experiment No.	Data.	Observed values after exposures of duration indicated.						
61	Exposure, *min*.....	0.75	1.5	2.58	4.13	6.0	9.08	15.0
	Efficiency, *per cent*.....	85.9	83.3	78.0	74.1	62.4	50.8	36.3
	k.....	0.089	0.053	0.042	0.315	0.034	0.032	0.029
62	Exposure, *min*.....	0.75	2.5	4.0	6.0	9.0	13.0	22.0
	Efficiency, *per cent*.....	94.2	76.2	72.0	67.9	46.7	41.1	23.7
	k.....	0.035	0.047	0.036	0.028	0.037	0.03	0.028
64	Exposure, *min*.....	0.75	2.5	4.0	6.08	9.0	0.3	21.0
	Efficiency, *per cent*.....	97.4	91.0	83.7	76.3	66.7	55.4	33.2
	k.....	0.016	0.016	0.019	0.019	0.02	0.02	0.023
67	Exposure, *min*.....	1.0	2.5	4.0	6.0	9.0	14.0	20.75
	Efficiency, *per cent*.....	93.2	84.5	76.3	66.6	54.3	39.0	19.7
	k.....	0.031	0.03	0.03	0.03	0.03	0.029	0.034
69	Exposure, *min*.....	0.55	1.0	2.5	4.0	7.0	11.0	18.0
	Efficiency, *per cent*.....	110.7	114.6	86.4	63±*	54.9*	52.5	28.9
	k.....	—	—	0.026	0.048	0.037	0.026	0.03
70	Exposure, *min*.....	0.67	1.33	3.0	6.0	10.0	16.0	24.0
	Efficiency, *per cent*.....	90.3	86.4	75.4	71.3	52.5	31.8	15.7
	k.....	0.067	0.048	0.041	0.025	0.028	0.031	0.033
71	Exposure, *min*.....	1.0	2.0	3.5	6.0	10.0	16.0	25.0
	Efficiency, *per cent*.....	97.8	91.0	82.4	71.5	52.4	37.7	25.6
	k.....	0.01	0.021	0.024	0.024	0.028	0.026	0.024
72	Exposure, *min*.....	0.5	1.0	2.0	4.0	7.0	10.0	18.0
	Efficiency, per *cent*.....	95.7	90.9	85.6	74.0	57.6	43.6	23.6
	k.....	0.04	0.042	0.034	0.033	0.034	0.036	0.034
Mean.	Exposure, *min*.....	1.0	2.0	4.0	7.0	10.0	15.0	20.0
	k.....	0.026	0.032	0.03	0.028	0.03	0.028	0.028

* Doubtful.

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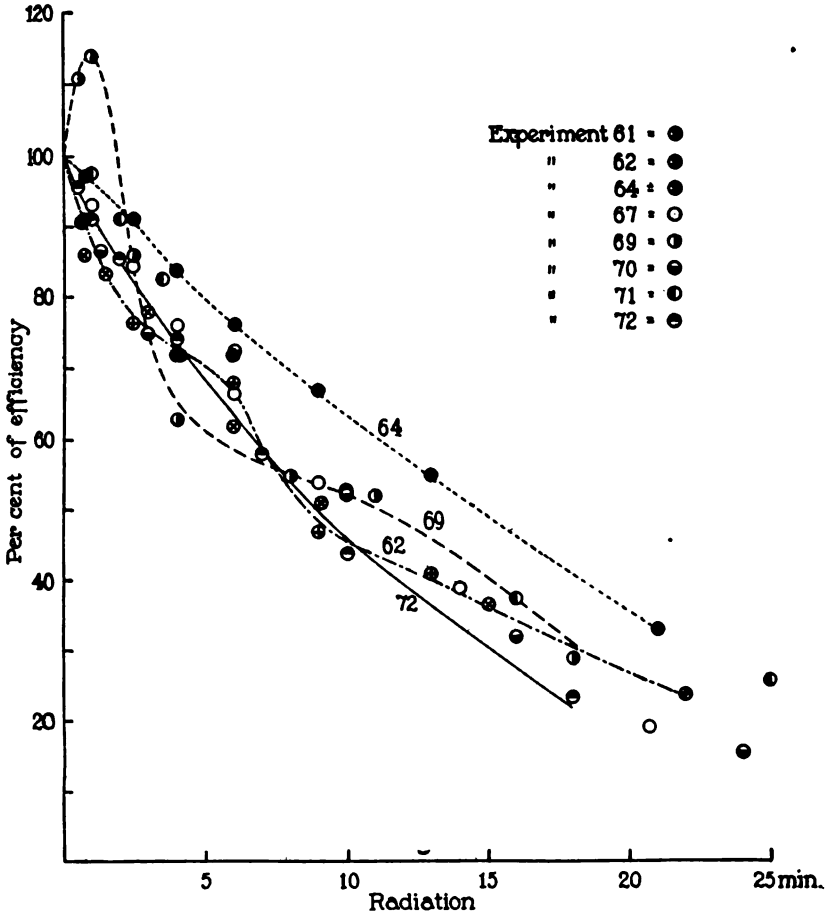

FIG. 2. Courses of the process of photoinactivation of eight samples of complement. Curves are drawn to show four characteristic examples. The ordinates represent efficiency in per cent of that of unradiated complement, and the abscissæ represent time of exposure to light in minutes.

study of a larger number of sera, yet those here presented will serve to demonstrate two facts; first, that the primary photoreaction is monomolecular, and second, that upon this primary monomolecular reaction there are superimposed accessory processes which serve to increase or decrease the hemolytic power of the serum.

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Examination of the horizontal columns in Table IV headed k will show that the values in any one column are essentially constant. This is what is to be expected in case the course of the process is that of a monomolecular reaction, the equation of whose reaction curve or isotherm is $\frac{1}{t} \log \frac{a}{a-x} = k$, where a is the original concentration of the reacting substance, x its concentration after the time t, and k a constant. When the amount of injury is still very slight the errors of titration may cause relatively great variations of this value such as are to be found in Experiments 62 and 70 (Table IV).

In Fig. 2 the probable error of titration is less than the diameter of the circles surrounding each point. It is at once apparent that errors in titration will not account for the irregularity of some of the observed curves. But it is evident that such deviations from the course of a monomolecular reaction are not an essential part of the process of photoinactivation, because the average of all the curves is almost exactly the theoretical monomolecular reaction isotherm (Table IV). Only during the first 2 minutes, when the influence of errors in titration is very great, does the observed value of $\frac{1}{t} \log \frac{a}{a-x}$ for the average curve deviate from its mean value, 0.0289, by more than a fraction of its probable error, 0.0013.

Photoinactivation is therefore a process which goes on at a rate proportional to the concentration of a single disappearing molecular species, which we may consider to be the substance responsible for the hemolytic property of serum. The deviation of the observed courses of photoinactivation from monomolecular curves also demands explanation, but may best be considered in connection with evidence which will be presented in a subsequent paper.

SUMMARY.

The photoinactivation of complement has been studied with a view to determining if possible how many kinds of molecules disappeared during the reaction. It was found that:

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1. The apparent course of photoinactivation is that of a monomolecular reaction.

2. Diffusion is not the limiting factor responsible for this fact, because the temperature coefficient of diffusion is much higher than that of photoinactivation (Q_{10} = 1.22 to 1.28, and Q_{10} = 1.10 respectively).

3. There is no change in the transparency of serum solutions during photoinactivation, at least for light of the effective wave-length, which is in the ultra-violet region probably at about 2530 Ångström units.

It is pointed out that under these conditions only one interpretation is possible; namely, that during photoinactivation a single disappearing molecular species governs the rate of reaction. This substance must be primarily responsible for the hemolytic power of serum when it is used as complement.

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THE MECHANISM OF COMPLEMENT ACTION.

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It has been shown in the preceding paper that the hemolytic action of serum is to be attributed primarily to a single substance which is destroyed under the influence of ultra-violet light by a monomolecular reaction. This fact immediately raises the question of the nature of the photosensitive substance, and leads the writer to make some suggestions as to what such a substance might be.[1]

I.

Certain experiments of the writer seem definitely to exclude the possibility that the serum proteins are primarily responsible for the power of serum to act as complement in specific serum hemolysis, for this power can be abolished by the action of light without producing the sensitization to heat which is characteristic of the effect of light on serum proteins. These experiments, which are summarized in Table I, show that radiated complement is not thereby made more susceptible to injury by heating.[2]

If the rates of heat inactivation of any individual serum before and after radiation are compared, it will be noted that the differences of efficiency are so small as to be attributable to errors in titration;

* The experiments upon which this paper is based were done by the writer as Research Fellow in the Harvard School of Tropical Medicine.

[1] In this paper the word "complement" will be used to denote any solution of serum used as one component in specific serum hemolysis (the other component being amboceptor); while that particular ingredient of serum primarily responsible for its hemolytic power is designated as the "lytic substance" or "lytic principle."

[2] All three experiments were done before the development of dependable methods of titrating complement, and a probable error of about ±3 per cent is to be expected.

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and furthermore that the average value of the rate before radiation is almost exactly the same as the average value after radiation. This shows that radiation does not sensitize complement to heat. Experiments conducted for another purpose incidentally supply further proof that at temperatures up to 37°C. radiated complement deteriorates at the same rate as it did prior to radiation.[3]

Now Bovie[4] and Chalupecky[5] independently pointed out that ultraviolet radiation sensitizes such proteins as egg white and lens protein

TABLE I.

Summary of Experiments on the Effect of Heating upon Radiated and Normal Complement. Velocities Calculated as $k = \frac{1}{t} \log \frac{a}{a-x}$.

Experiment No.	Radiation.		Heating.					Ratio of velocities, Radiated: Normal.
	Exposure.	Injury.	Sample.	Bath temperature.	Exposure.	Injury.	Velocity.	
	min.	*per cent*		°C.	*min.*	*per cent*		
17	2	24	Radiated.	55.5	3	23.5	0.1165	1.25
			Normal.	55.5	3	19.3	0.0935	
34	45	87	Radiated.	52.0	11	84.0	0.77	1.23
			Normal.	52.0	11	76.0	0.624	
42	15	44	Radiated.	45.2	6	5.0	0.0056	0.55
			Normal.	45.2	6	9.0	0.0102	
Average								1.01

to subsequent heating, so that they coagulate much more rapidly or at lower temperatures. In Bovie's experiments radiated egg white coagulated even at room temperature. Schanz[6] showed that ultraviolet light has a similar effect on serum proteins; he also confirmed and extended the findings of Chalupecky on egg white and lens proteins.

If the hemolytic power of the serum were due to its protein content, radiation sufficient to destroy a large part of that power should be

[3] Brooks, S. C., *J. Med. Research*, 1920, xli, 411.
[4] Bovie, W. T., *Science*, 1913, xxxvii, 373.
[5] Chalupecky, H., *Wien. med. Woch.*, 1913, lxiii, 1986.
[6] Schanz, F., *Arch. ges. Physiol.*, 1915, clxi, 384.

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accompanied by changes which are at least qualitatively like those displayed by the pure proteins themselves under radiation. Since the lytic principle can be nearly destroyed without any perceptible alteration of the serum proteins, we are forced to the conclusion that complement is not one of the serum proteins, and is, to a certain extent at least, independent of them.

II.

This must not be thought to mean that the hemolytic power of serum is wholly independent of proteins under all conditions. The experiments of Jacoby and Schütze[7] on the inactivation of complement by shaking, those of Michaelis and Skwirsky[8] on the effect of proteases on complement, and many others indicate the contrary.

To obviate confusion it may be well to point out that while the hemolytic substance is probably not a protein, as explained in the preceding section, this is not incompatible with the fact that complement may be profoundly affected by changes in the serum proteins; inactivation may be the direct effect of some agent, *e.g.* light, or it may be indirect and due to the effect of a change produced by the action upon serum proteins of such an agent as a protease. The serum proteins thus altered may then act upon the lytic substance.

The following experiments bear out this idea, since they suggest that acid may inactivate complement not by means of its effect upon the lytic substance itself, but by altering the state of the serum proteins.

It has long been known that complement could be made inactive by the addition of acid and alkali, and that if more than a small amount of acid or alkali was added the inactivation was not reversible by subsequent neutralization of the added reagent. The precise limits of pH value between which inactivation is still reversible have not heretofore been determined.

Fresh guinea pig serum was diluted to 20 volumes with 0.85 per cent NaCl solution[9] and kept in containers immersed in ice water

[7] Jacoby, M., and Schütze, A., *Berl. klin. Woch.*, 1909, xlvi, 2139.

[8] Michaelis, L., and Skwirsky, P., *Z. Immunitätsforsch., Orig.*, 1910, vii, 497.

[9] The balanced solution employed for dilution throughout all other parts of this work would have resisted changes in reaction by reason of its bicarbonate content, and hence could not be used in this particular operation.

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at all times except for one period of about ½ hour while the acid was acting; during this period a temperature of 10°C. was maintained. Three solutions were prepared for admixture with the complement: 0.04 N HCl, 0.04 N NaOH (adjusted by comparison with HCl), and a 0.02 N NaCl solution made by mixing equal volumes of these two solutions. All these solutions were cooled to the temperature of the diluted complement before mixing.

The general plan of the experiments involved the addition of the desired amount of acid, and after a certain length of time its neutralization by the equivalent amount of alkali. In each case enough 0.02 N NaCl was added to make the final volume and concentration the same in all the tubes. The effect of the change in NaCl concentration (from 0.85 to 0.73 per cent) was negligible in view of the subsequent dilution with much larger amounts of balanced solution. The dilution of the serum was taken into account in the calculations.

Sets of clean sterile test-tubes were set in an ice water bath, each set consisting of a tube in which was placed 10 cc. of 5 per cent complement, and tubes for the acid, alkali, and NaCl solutions. At the beginning of the experiment the samples of complement were in turn poured into the tubes containing acid and quickly mixed by pouring back and forth twice. After the desired interval these mixed samples were poured in like manner into the tubes containing alkali, and then into the final tube containing NaCl to equalize the volumes. The same operations were performed even when no acid or alkali was to be added, to obviate any possible effect due to the slight foaming involved in mixing the samples even though this caused no demonstrable inactivation.

After this treatment the complement was further diluted to the desired extent and titrated as described in a previous paper,[10] by a method in which the probable error of determining the relative efficiency of samples of complement lies between 1 and 2 per cent.

The action of the acid apparently takes place very quickly; certainly in less than 15 minutes at 0°C. A preliminary experiment will serve to demonstrate this point. The efficiencies of two samples of complement left in the acid for 15 and for 66 minutes respectively

[10] Brooks, S. C., *J. Med. Research*, 1920, xli, 399.

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were 88.6 and 89.7 per cent respectively. These figures differ by considerably less than the probable error and are therefore to be considered identical. In the remaining experiments the acid was allowed to act for about 30 minutes (the time varying by not more than ½ minute in any one experiment) so that there could be no doubt that it would have its full effect.

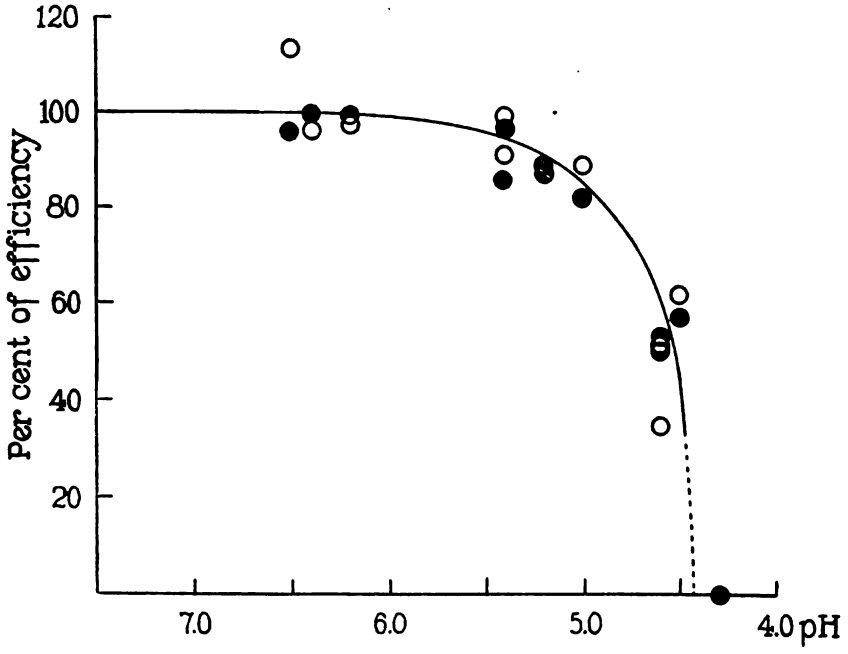

FIG. 1. The effect of temporary acidulation upon the subsequent efficiency of radiated and normal complement. The ordinates represent efficiency in per cent of that of the corresponding unacidulated complement, and the abscissæ represent the pH to which the complement was exposed during the period of acidulation. Open circles, normal complement; solid circles, radiated complement; where these coincide the circles are shaded.

The relation between the true reaction during acid treatment and the hemolytic activity after the restoration of the normal reaction of serum (pH = 7.6) will be most easily grasped by a study of Fig. 1 in which relative efficiencies are plotted as ordinates against hydrogen ion concentration in terms of pH. These values of pH are accurate

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only within 0.2 pH units, because they were judged only on the basis of the approximate colors, in samples of the acidulated complement, of two or more of the following indicators; phenol red, brom cresol purple, methyl red, and brom phenol blue. No standard comparison solutions were used. Bearing these facts in mind it will be seen that the experiments agree in showing very little change in the efficiency of complement (perhaps even a slight increase in some cases) as long as the hydrogen ion concentration is less than pH = 6.0; when the

TABLE II.

The Permanent Effect of Temporary Acidulation on Radiated and Unradiated Complement.

Experiment No.	Sample.	Exposure to light.	Efficiency normal = 100 per cent.	Exposure to acid.	Efficiency at indicated pH; referred to corresponding unacidulated sample as 100 per cent.					
					pH	Efficiency.	pH	Efficiency.	pH	Efficiency.
		min.		*min.*		*per cent*		*per cent*		*per cent*
90	Normal.	0	100.0	33	6.5	113.1	5.4	91.0	4.3	0.0
	Radiated.	4	76.3	32	6.5	95.8	5.4	85.8	4.3	0.0
96	Normal.	0	100.0	31	5.4	99.2	5.0	87.2	4.6	33.8
	Radiated.	5	92.7	31	5.4	95.5	5.0	82.4	4.6	53.0
97	Normal.	0	100.0	31	6.4	96.3	5.2	86.9	4.6	50.7
	Radiated.	5	73.1	31	6.4	99.3	5.2	87.1	4.6	50.5
98	Normal.	0	100.0	35	6.2	96.8	5.2	88.8	4.5	61.6
	Radiated.	6	86.7	35	6.2	98.6	5.2	88.8	4.5	56.6

pH = 5.5, there is some decrease in the hemolytic power, and as it exceeds 5.0 there is a rapid drop in efficiency, complete inactivation being the result at pH = 4.3. The data for these experiments are given in detail in Table II.

There is a remarkable parallelism between this behavior and the known characteristics of the serum proteins. The serum proteins are usually classified as eu- and pseudoglobulins and albumins according to their solubility in water and in salt solutions; but there are no sharp divisions between these groups. The isoelectric point of euglobulins as prepared by Rona and Michaelis[11] is given by them as 3.6

[11] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

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$\times 10^{-6}$, which is equivalent to a pH of about 5.4; that is, the point at which complement begins to be affected by acid. The hydrogen ion concentration at which the inactivation of complement becomes complete is about pH = 5.0; this is just on the acid side of the isoelectric point of the serum albumin, which is at pH 4.7.[12]

Now amphoteric substances, among which we must number the serum proteins, behave as anions on the alkaline side of their isoelectric points, and as cations on the acid side of their isoelectric points; while at this point the sign of their charge changes from negative to positive, and there are great changes in their physical properties corresponding to slight changes of hydrogen ion concentration.[13]

TABLE III.

The pH of Samples of the Same Lot of 5 Per Cent Complement Radiated for Different Lengths of Time.

Exposure.	Efficiency.	Hydrogen ion concentration.
min.	*per cent*	pH
0	100.0	7.55
½	95.7	7.52
1	90.9	7.52
2	85.6	7.52
4	74.0	7.55
7	57.6	7.50
10	43.6	7.55
18	23.6	7.55

Since the hydrogen ion concentration at which these changes occur is coincident with that at which complement loses its hemolytic power, it is not improbable that there is a connection between the phenomena. We might suppose that the protein cation takes part in an irreversible reaction with the lytic substance or some one of its constituent groups. Other possibilities might be suggested, but the evidence is not adequate for distinguishing between one possibility and another, and the essential point remains the same; that there is some close connection between the state of the serum proteins and the effectiveness of the lytic substance.

[12] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxiii, 456.
[13] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

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If this is true then the lytic substance may be inactivated in either of two ways: either directly, as by ultra-violet light; or indirectly by means of agents affecting other serum constituents; *e.g.*, the proteins[14] (Table III).

These same experiments show also that radiation sufficient to destroy the hemolytic power of serum may fail to have any appreciable effect on the serum proteins; otherwise there should be a difference between radiated and normal complement in their susceptibility to inactivation by acids (Fig. 1 and Table II). Differences between radiated and normal complement do occur occasionally, but they are so irregular as to have no obvious general significance. Radiation fails to sensitize complement to either heating (Section I) or acidulation, and the two sets of experiments substantiate each other in this respect.

III.

In view of the fact that surface tension of complement has been thought by some[15, 16] to be connected directly with its hemolytic power it is interesting to note the change in surface tension taking place when complement is inactivated by ultra-violet light.

The surface tension of 5 per cent complement solution was measured by means of a Traube stalagmometer and was found to decrease slightly (Table IV and Fig. 2). Measurements of the time of outflow made at the same time are probably not reliable because of the presence of occasional wisps of cotton in the solutions, but since they seem to indicate a change in viscosity they are also given: if there is any definite change of viscosity it is a decrease.

[14] In this connection it should be pointed out that changes in hydrogen ion concentration play no part in the ordinary course of photoinactivation. Samples of 5 per cent complement were taken immediately after radiation for various lengths of exposure and their hydrogen ion concentration determined by the addition of an appropriate amount of phenol red and comparison of the resulting color with that produced in solutions of known hydrogen ion concentration. The least active sample had been reduced to a relative efficiency of 23.6 per cent. There was an irregular variation of between pH = 7.55 and 7.50, which is so small as to be utterly negligible (Table III). The complement is at all times exposed to a hydrogen ion concentration the same as that prevailing in the blood plasma.

[15] Traube, J., *Biochem. Z.*, 1908, x, 371.

[16] Traube, J., *Biochem. Z.*, 1908, x, 380.

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TABLE IV.

The Surface Tension and Viscosity of 5 Per Cent Complement Solutions Radiated for Different Lengths of Time.

Experiment No.	Exposure.	Efficiency.	Outflow from stalagmometer.		
			No. of drops.	Time of outflow	
	min.	*per cent*		*min.*	*sec.*
71	0	100.0	56.4	5	17
	2	91.0	56.4	5	5
	3½	82.4	57.7	5	13
	10	52.4	57.8	4	45
	Diluent alone.		56.0	4	53
72	0	100.0	56.3	5	2
	½	95.7	57.1	4	59
	2	85.6	57.2	4	54
	8½	50.0	57.25	4	46
	Diluent alone.		56.1	5	5

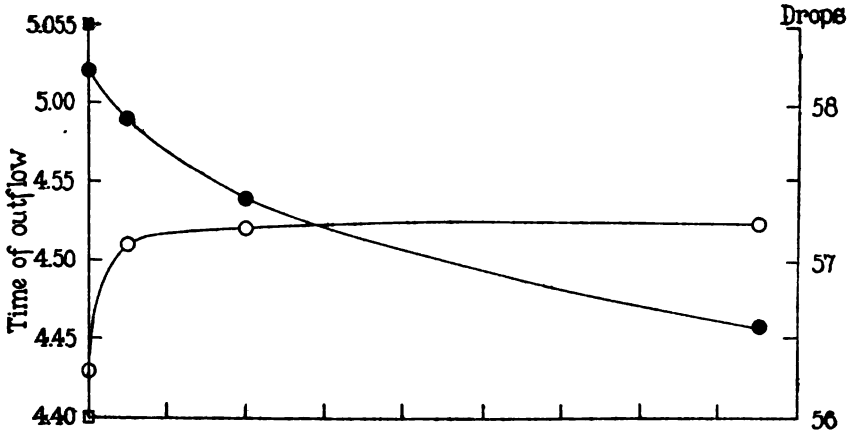

FIG. 2. The progressive changes in surface tension and viscosity of complement during photoinactivation. The squares indicate the data for the diluent alone. The ordinates represent the time, or the number of drops required for the outflow of a constant amount of complement from a Traube stalagmometer. The abscissæ represent in minutes the time of exposure of the samples to the light. Open circles and square, surface tension; solid circles and square, viscosity.

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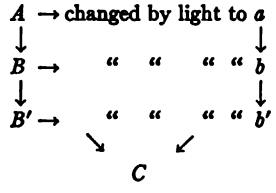
The change of surface tension is like that accompanying thermo-inactivation,[17] and contrary to the hypothesis advanced by Traube,[15, 16] is probably not the cause of the change in hemolytic power.[17]

IV.

In the following paragraphs there is suggested an hypothesis which has the advantage of explaining many of the known properties of serum complement with more definiteness than current theories and without being in conflict with any well established fact. Definite substances are named only for the sake of making it easier to grasp certain essential ideas, and not with any pretense that complement must be supposed to consist of just these particular substances. The main ideas are these: that there is a hemolytic substance in serum which is constantly breaking down into non-lytic material, and constantly renewed from a store of some "precursor" substance; that this lytic substance is so related to the serum proteins that it is more or less permanently inactivated by certain changes in the serum proteins; and that the hemolytic substance and all its precursors contain a certain photosensitive molecular grouping whose alteration results in photoinactivation.

These essential points may be embodied in the following scheme



in which the different letters represent different chemical individuals; the changes $A \rightarrow B$, $a \rightarrow b$, $B' \rightarrow C$, and $b' \rightarrow C$ represent hydrolyses, and the changes $B \rightarrow B'$ and $b \rightarrow b'$ the passage of B or b from solution in fats to solution in water as a result of a change in relative solubility produced by the preceding hydrolysis. B (*i.e.* 'B') is the principal lysin, and b', formed by radiation, is probably also hemolytic to a certain extent.

When complement is heated we need consider only the left-hand series, $A \rightarrow B \rightarrow B' \rightarrow C$. A is a precursor present in large amount

[17] Schmidt, H., *J. Hyg.*, 1913, xiii, 314.

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in serum, and undergoing transformation into B at a rate which is not very greatly accelerated by an increase in temperature. The change $B \rightarrow B'$ is very rapid and is probably never a limiting factor. B' is the lysin and undergoes hydrolysis at a rate markedly influenced by changes of temperature, and proportional to the amount of B' present at the moment, as in any monomolecular reaction such as most hydrolyses.

Let us suppose serum to be collected and placed at 0°C. The breakdown of B' may be retarded by the lowered temperature, and B' will accumulate until there is so much that the amount broken down in a unit of time will equal the amount formed from A. (We may suppose the process $B \rightarrow B'$ to be so rapid as to have no perceptible influence on variations in the amount of B'.) Since the reaction $A \rightarrow B$ is also monomolecular, the amount of B formed in any time interval will be less and less as A is used up; and to keep pace with this change B', the lysin, must also gradually decrease in amount. Thus the hemolytic power, which is proportional to the amount of B', will gradually decrease. This is a well known phenomenon.

Let us suppose that serum which has reached this steady state of equal formation and destruction of B' is suddenly raised to a high temperature, *e.g.* 56°C.; the destruction of B', which we have assumed to have a high temperature coefficient, will be enormously accelerated while its formation will still be relatively slow. As a result the hemolytic power of the serum will rapidly decrease, and, since its rate of formation is at first relatively negligible, the decrease will follow the course of a monomolecular reaction. This will continue until so little B' is left that it decomposes at a rate comparable with that of the change $A \rightarrow B$. Madsen and Watabiki[18] present data which not only show that thermoinactivation of complement follows the course of a monomolecular reaction (about as closely as photoinactivation) but that the temperature coefficient at 50–56°C. is very high (Q_{10} lying between 123 and 366.8), but between 3 and 37°C. is about that of typical dark reaction (Q_{10} between 1.98 and 2.94).[19]

[18] Madsen, T., and Watabiki, T., *Oversigt. kong. danske Videnskab. Selskabs Forhandl.*, 1915, 125.

[19] Madsen and Watabiki express their results in terms of the temperature coefficient of the van't Hoff-Arrhenius formula. The values of Q_{10} given above are calculated directly from their data.

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This agrees exactly with our hypothesis, both as to the order of the reaction of thermoinactivation, and in that the destruction of B' with its high temperature coefficient is predominant at temperatures above 50°, while the limiting reaction at temperatures below 37° is the breakdown of A which has a relatively low temperature coefficient. The gradual decrease of B' then keeps pace with the gradual decrease of A. Whenever, as after brief heating to 56° for example, the concentration of B' is disproportionately reduced and a large amount of A is still left, the latter will act as a reservoir from which B' will be restored to the concentration appropriate to the temperature and the remaining amount of A. The hemolytic power being proportional to B' will be low immediately after heating and will then be regenerated as Gramenitzki[20] and the writer[1] have shown to be the case.

Turning now to the question of what occurs during photoinactivation we find that if the time curves of several experiments are averaged the process follows the course of a monomolecular reaction.[21] Now if only B' were hemolytic and the members of the left-hand branch of our hypothetical series (*i.e.* A, B, and B') contained the same photosensitive group, the proportion of each substance destroyed would be the same; that is, one-tenth or one-half or nine-tenths of each of them would be destroyed, but not one-half of A and nine-tenths of B and B'. Under certain conditions which were defined in a previous paper[21] the destruction of a substance by light will follow the course of a monomolecular reaction, and if B' is so destroyed, and no disproportionate amount of A and B is left (the latter case occurs when complement is briefly heated), then there can be no regeneration. The theory then accounts for the observed course of photoinactivation and for the fact that no regeneration follows photoinactivation.

The reader should now note carefully the curves shown in Fig. 2, of the preceding paper. They are all characterized by the fact that at some time during the process the hemolytic activity exceeds that to be expected if the process followed exactly the course of a monomolecular reaction; there is a "wave" of excess activity. If we suppose that b' is hemolytic and that it forms and decomposes at a relatively

[20] Gramenitzki, M., *Biochem. Z.*, 1912, xxxviii, 501.

[21] Brooks, S. C., *J. Gen. Physiol.*, 1920–21, iii, 169.

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rapid rate it is easy to see that as A is changed to a the concentration of a will increase; later as A is used up a will decrease again. Similarly b and b' will appear, increase, and disappear. If b' is hemolytic there will be a "wave" of hemolytic power in addition to that due to B' and not only the average time curve of photoinactivation, but also the divergence of individual curves from this average is satisfactorily accounted for. The time at which this wave occurs in the course of the process will depend on the rate of the slower of the processes, a to b and b to b'; presumably this is a to b which may well be dependent on an enzyme whose concentration is different in different samples of serum. If the conditions are favorable we may expect the wave of excess hemolytic power to be so large and so early that the complement becomes more efficient when radiated, as during the early part of Experiment 69 of the preceding paper.[22]

In order to render this hypothesis more tangible it is desirable to suggest the nature of the substances A, B, a, b, and so on, even if there is no direct evidence for their exact composition. There is considerable evidence that fatty acids are important in immune reactions: Warden[23] has synthesized from fatty acids antigens which produce specific antibodies against blood cells and gonococcus; Jobling and Bull[24] have shown that the lipase content and hemolytic power of human sera vary together. Noguchi,[25] among others, has shown striking analogies between complement and solutions of fatty acid compounds dissolved in serum albumin solutions. All these facts suggest the presence of a fatty acid compound acting as hemolysin in complement.

The most active hemolysin of which the writer has found any record is the "lysocithin" produced by the action of cobra venom upon crude lecithin and studied by Fourneau and his students. It appears to be at least 50 times as active as saponin and perhaps 100 times as active as the most hemolytic soaps. It is a fatty acid compound: choline monopalmitoglycerophosphate.[26] It should be noted that

[22] Brooks, S. C., *J. Gen. Physiol.*, 1920–21, iii, 180, Table IV; and 181, upper curve of Fig. 2.

[23] Warden, C. C., *J. Infect. Dis.*, 1918, xxiii, 504.

[24] Jobling, J. W., and Bull, C. G., *J. Exp. Med.*, 1913, xviii, 61.

[25] Noguchi, H., *Biochem. Z.*, 1907, vi, 327.

[26] Fourneau, E., and Delezenne, C., *Bull. Soc. Chim.*, 1914, series 4, xv, 354.

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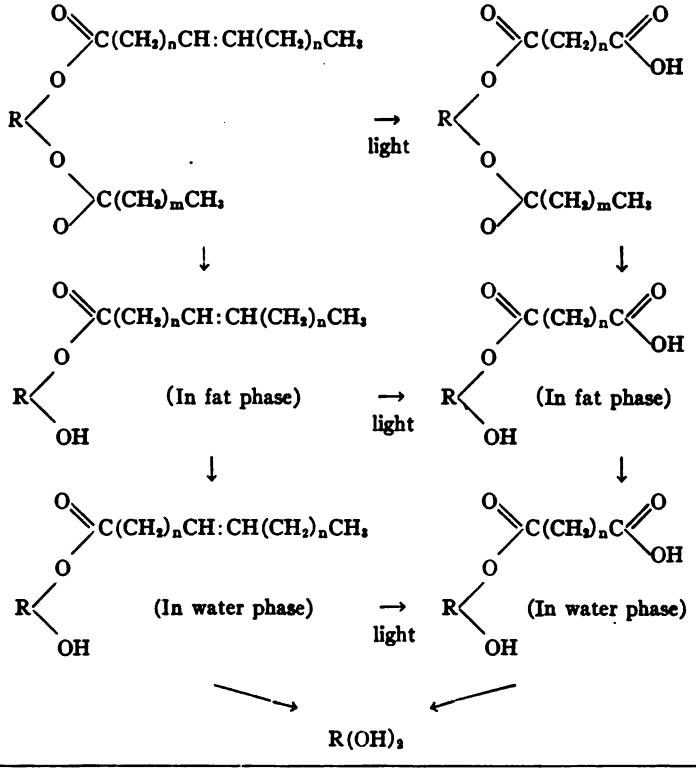
this substance is soluble in warm water, and hardly soluble in benzene, while complement is an aqueous solution, and is not easily injured by extraction with benzene.[27] Lysocithin also forms an irreversible compound with emulsified cholesterol, thus reminding one of the fact that emulsions of cholesterol "fix" complement. Furthermore, lysocithin is formed by the action of venom lipase on lecithin but is destroyed by the further action of the same agent; removal of the second fatty acid leaves the lecithin complex inactive.[28] These two steps might be compared with the process which results in spontaneous deterioration of complement.

With these facts in mind we may proceed to picture the nature of the hemolysin system in complement as follows:



[27] Schmidt, P., *Z. Chem. Ind. Koll.*, 1912, xi, 5.

[28] Delezenne, C., and Fourneau, E., *Bull. Soc. Chim.*, 1914, series 4, xv, 421.

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In this scheme R represents choline glycerophosphoric acid or some similar substance, and, since compounds of lower fatty acids are markedly lacking in hemolytic power, the two fatty acids may each be supposed to contain at least ten carbon atoms. One of the acids is for two reasons supposed to be unsaturated: because compounds of unsaturated fatty acids are in general more hemolytic than those of the corresponding saturated acids, as shown by Lamar;[29] and because this furnishes a molecular grouping known to be attacked by light[30] with a consequent break in the fatty acid chain which might be expected to bring about a great decrease in hemolytic power.[31]

Many phenomena displayed by complement are undoubtedly dependent in some way upon the physical or chemical state of the serum colloids, as is shown for example in the previously noted inactivation by acid. Sachs and Stilling[32] have shown that inulin suspended in cold water affects complement, while if first dissolved by warming the water it no longer has any effect. It is not surprising then that complement is inactivated by shaking, since shaking, or rather the attendant foaming, causes irreversible coagulation of proteins,[33] nor that processes which precipitate serum globulins should, if the process is reversible, produce the so called "fractions" which upon being recombined regain their hemolytic power. The conflicting nature of the evidence about these so called fractions, as well as the writer's own experience with several methods of obtaining the fractions, leads him to doubt the uniformity of the preparations secured by different investigators. Evidently the lytic substance is usually held inactive in one of the fractions, since exposure of sensitized red blood cells to the globulin or "mid-piece" fraction results in a change which makes them susceptible of lysis by the albumin fraction, which is therefore supposed to combine with some element in the red blood cells indirectly through the "mid-piece." It is for this reason that the albumin fraction has received the name "end-piece."

[29] Lamar, R. V., *J. Exp. Med.*, 1911, xiii, 380.
[30] Ciamician, G., and Silber, P., *Ber. chem. Ges.*, 1914, xlvii, 640.
[31] Shimazono, J., *Arch. exp. Path. u. Pharmakol.*, 1911, lxv, 361.
[32] Sachs, H., and Stilling, E., *Z. Immunitätsforsch., Orig.*, 1917, xxvi, 530.
[33] Ramsden, W., *Arch. Physiol.*, 1894, 517.

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When cobra venom is allowed to act upon complement under certain conditions there ensues a change in the serum such that when this cobra serum, as it is called, is combined with either mid- or end-piece of normal complement, or when either fraction of the cobra serum is combined with the supplementary fraction of normal serum the mixture is hemolytic.[34] This is supposed to be due to the action of cobra venom upon a "third component" of complement. Hemolysis by complement would then be considered to result from the combined action of mid-piece, end-piece, and third component, all of which must be present.

The writer is inclined to regard the cobra serum as serum from which all the lysin and its precursors have been removed. The third component, which is destroyed by cobra venom, is the lysin and its related substances. This is in accord with the work of Delezenne and Fourneau[28] and with the observed characteristics of cobra serum.

Phenomena susceptible of explanation in terms of the scheme here proposed abound in the literature, and their descriptions might be multiplied indefinitely if anything were to be gained by so doing. It is of greater significance that search of the voluminous literature on complement has so far failed to discover any well established fact which is incompatible with this hypothesis. If such facts are found, the hypothesis must of course be replaced by some better one; but the writer believes that the main ideas underlying it, namely successive transformations of precursor into lysin and then into inactive products, and the dependence of this lysin on the serum proteins, will prove to be valid. At any rate they seem preferable to certain prevailing explanations based upon the rather indefinite concept of a hemolytic power resulting from the colloidal properties or "lability" of serum proteins.[35]

SUMMARY.

It has been shown:

1. That complement exposed to ultra-violet light is not thereby sensitized to the action of heat (which indicates that it is not protein).

[34] Ritz, H., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 62.

[35] Such ideas have been expressed by many investigators and have recently been developed and emphasized by Sachs in a review of the work of his laboratory (Sachs, H., *Kolle Z.*, 1919, xxiv, 113).

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2. That inactivation of complement by ultra-violet light is accompanied by a decrease in its surface tension.

3. That photoinactivation of complement is not a result of any changes in hydrogen ion concentration since these are less than 0.05 pH.

4. That hydrogen ion concentrations high enough to transform serum proteins from the cation to the anion condition (*i.e.* past the isoelectric point) permanently inactivate complement.

These facts together with those given in previous papers lead to the following hypotheses.

1. That there is present in serum a hemolytic substance which is formed from a precursor (which may resemble lecithin) and is constantly being formed and simultaneously being broken down into inactive products.

2. That both precursor and lysin contain the same photosensitive molecular group.

3. That the lytic substance is dependent for its activity upon the state of the serum proteins.

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COMPARATIVE STUDIES ON RESPIRATION.

XIII. An Apparatus for Measuring the Production of Minute Quantities of Carbon Dioxide by Organisms.

By MARIAN IRWIN.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, June 28, 1920.)

Osterhout[1] has described an apparatus by means of which the production of small quantities of carbon dioxide by organisms may be measured at short intervals. In this apparatus air is caused to circulate through a tube containing the organisms and then into a tube containing an indicator, the color changes of which are compared with buffer solutions containing the same amount of indicator.

The purpose of the present article is to describe a modified apparatus devised by the writer.

The whole apparatus, with the exception of a short piece of rubber tubing, is made of glass. In order to prevent leakage the joints and glass stop-cocks are ground with special care.

Fig. 1 shows a sectional view of a portion of the apparatus. The tube O contains the organisms and the tube I contains an indicator. These tubes are connected to the apparatus by ground joints. When the stop-cock S is opened, and the stop-cocks S_1 and S_2 are closed,[2] the air is allowed to pass from the tube O containing the organisms to the tube I containing the indicator.[3] When S is closed and S_1 and S_2 are opened, the air passes from O to I through N which contains lumps of sodium hydroxide for the absorption of carbon dioxide. The ground joint J allows the apparatus to be disconnected.[4] The dotted

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

[2] A single three-way stop-cock may be used in place of S, S_1 and S_2 in order to avoid dead spaces.

[3] The indicator tube and the inlet tube within it are made of Pyrex glass.

[4] It may be desirable to make the entire apparatus of Pyrex glass in which case the ground joint J may be transferred to the right of S. This facilitates the substitution of tubes of various sizes in place of O.

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lines at X and Y indicate two glass tubes running off at right angles and connecting (at the points marked X_1 and Y_1 in Fig. 1, *B*) with another tube, composed partly of glass and partly of rubber. The central portion of this tube (R, Fig. 1, *B*) consists of a piece of rubber tubing[5] 2 mm. in thickness and 7 mm. in diameter (inside) which is joined to glass tubes at W and Z. The ends of the glass tubes are about 1 inch apart. In order to avoid leakage, the rubber tubing is

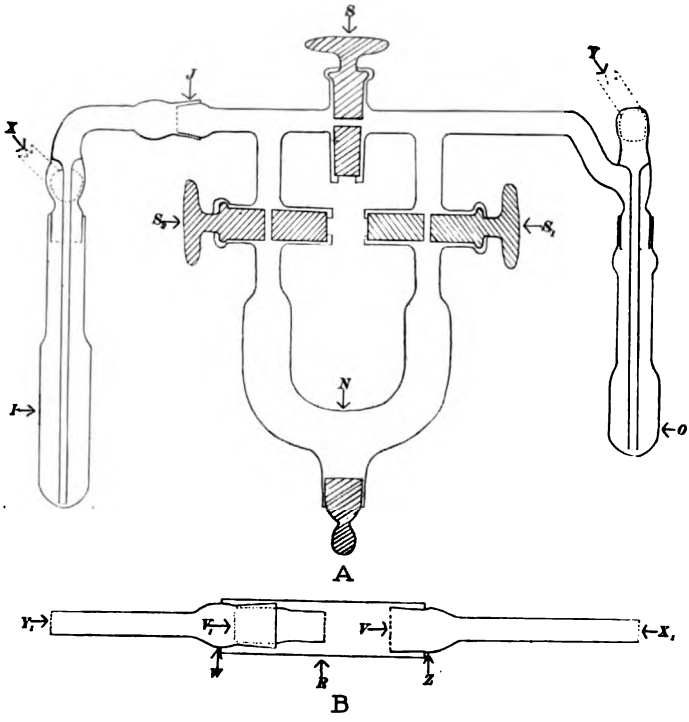

FIG. 1. (*A*) Apparatus for measuring the production of CO_2. Organisms are placed in the tube O and the CO_2 is carried over into the tube I which contains an indicator. The tube N contains NaOH for absorbing CO_2 when this becomes necessary.

(*B*) The rubber tube R is compressed at intervals, forcing air through the valves V and V_1 and so maintaining a circulation throughout the apparatus.

[5] Experiments are in progress with a view to replacing the rubber by other materials.

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slipped over the glass for about 1 inch and cemented to the glass by soft de Khotinsky cement. V and V_1 show the position of the valves, which allow the air to pass in one direction only (from Z to W). The valves are made of thin rubber sheeting attached to perforated rubber stoppers.

In order to produce a circulation of air the piece of rubber tubing is alternately compressed and released by means of the device shown in Fig. 2. The motor operates a cam the turning of which causes the

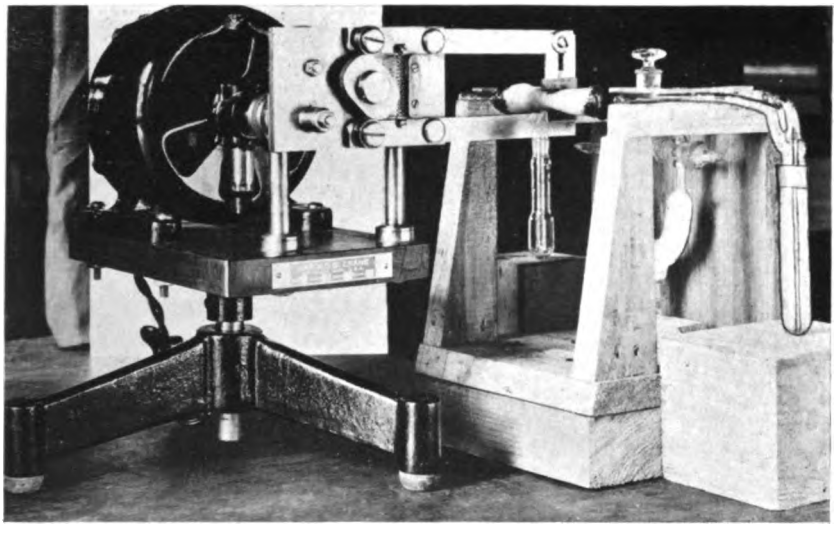

FIG. 2. The motor turns a cam which causes the horizontal arms alternately to approach and separate, thus compressing a piece of rubber tubing and maintaining a circulation of air in the apparatus.

two horizontal arms alternately to separate and to approach each other (they are held together by a vertical spring at the right of the cam[6]). Two projections at the ends of these arms are adjusted so as to compress the rubber tubing to the proper degree. The arms are made so that the height and the angle can be adjusted. The writer prefers a speed of 120 compressions per minute (the speed of the motor is reduced by gears).

[6] A worm may be substituted for the cam if noiseless operation is desired.

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The glass portion of the apparatus is rigidly fastened to the wooden frame by two screws. The wooden frame, however, is not fastened to the table but is allowed to move freely to compensate for the slight motion produced by the compression of the rubber tube; without this freedom of motion the connecting glass tubes might be broken.

When a constant temperature must be maintained it may be desirable to immerse the apparatus in water. The motor must then be adjusted so that the arms which compress the rubber tubing occupy a vertical position. The water should be contained in a suitable glass vessel so arranged that the changes in the color of the indicator may be readily observed. The buffer solutions must also be immersed and should be placed beside the tube I which contains the indicator.

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ENVIRONMENTAL FACTORS OTHER THAN TEMPERATURE AFFECTING FACET NUMBER IN THE BAR-EYED MUTANT OF DROSOPHILA.*

By JOSEPH KRAFKA, Jr.

(From the Zoological Laboratory of the University of Illinois, Urbana.)

(Received for publication, August 5, 1920.)

In a recent paper the author[1] has undertaken to show the relation between temperature and the facet number in the bar-eyed mutant of *Drosophila melanogaster* Meig. Since it was essential to know the part played by other environmental factors the experimental evaluation of some of these was carried out. While these experiments were not as critical as was desired, they give some idea as to the respective value of food, humidity, and evaporation as these are commonly represented in *Drosophila* culture technique. The following data are submitted, then, not as a final analysis, but rather as a basis for further work.

The work in all cases was carried out on the Ultra-bar mutant. For a direct comparison the data on the effect of temperature are given in Table I. The temperature data are for the interval 23–29°C., as the experiments involving the other factors were carried out at these temperatures.

An interesting suggestion for further work appeared in the analysis of the few experiments dealing with different kinds of foods. Since consistent results were obtained in three successive experiments it may be that they have some significance.

It is the experience of nearly all investigators rearing *Drosophila*, that fermented banana rapidly becomes acid at the high temperatures and that oviposition is much retarded. In an attempt to eliminate

* Contribution from the Zoological Laboratory of the University of Illinois, No. 165.

[1] Krafka, J., Jr., The effect of temperature upon facet number in the bar-eyed mutant of *Drosophila*, *J. Gen. Physiol.*, 1919–20, ii, 409, 433, 445.

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the acidity, I tried raising mass cultures on pure Fleischmann's yeast made into a paste and covered with cotton-wool. To my surprise the cultures hatched 1 day earlier than did the banana control and an examination of the facet counts showed an appreciable difference in

TABLE I.

Effect of Temperature upon Facet Number in the Ultra-Bar Mutant of Drosophila melanogaster.

Temperature at which flies developed.	Mean facet number. Females.	Difference in facets per °C.	Mean facet number. Males.	Difference in facets per °C.
°C.				
23	28.30±0.24		31.43±0.24	
		1.53		1.91
25	25.24±0.09		27.60±0.10	
		1.98		1.95
27	21.27±0.10		23.70±0.11	
		2.02		2.34
29	17.23±0.08		19.02±0.08	
Average.....		1.84		2.07

TABLE II.

Effect of Various Foods upon Facet Number in the Ultra-Bar Mutant of Drosophila melanogaster.

Food on which larvæ were reared.	Experiment 55. Temperature 23°C.		Experiment 61. Temperature 23°C.		Experiment 71. Temperature 27°C.	
	Females.	Males.	Females.	Males.	Females.	Males.
Fleischmann's yeast..	23.72±0.34	27.37±0.27	22.40±0.18	25.01±0.18	18.28±0.24	19.85±0.74
Yeast foam...	27.53±0.73	28.80±0.82	24.04±0.26	26.92±0.32		
Banana..	26.69±0.62	32.20±0.48	29.54±0.34	32.50±0.27	20.80±0.34	22.96±0.12

the mean. A repetition of the experiment at 23 and again at 27°C. gave consistent results. What the actual factor is that underlies this phenomenon is still uncertain. The data are given in Table II.

Humidity has been one of the factors to receive considerable attention in the past few years. Since the apparatus for humidity and

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temperature control were at hand several experiments were planned to test the action of that factor on development. Culture bottles, fitted up in the ordinary manner, were inclosed in humidity cases, one in which the humidity remained practically constant at 35 per cent, while the other was set for 60 per cent. Little or no difference was noted in the rate of development, or in the facet count (Table III).

Direct evaporation was next tried. Ordinary culture bottles were fitted with cork stoppers. A piece of glass tubing, extending through the cork to the surface of the banana, admitted air from the humidity control pipes. Another short tube with a cotton plug permitted

TABLE III.

*Effect of Humidity and Evaporation on Facet Number in the Ultra-Bar Mutant of Drosophila melanogaster.**

Atmospheric conditions under which immature stages were passed.	Mean facet number.		Range in facet number.	
	Females.	Males.	Females.	Males.
Culture bottle in 35 per cent humid air	26.59±0.32	28.86±0.37	21 to 32	23 to 38
Culture bottle in 60 per cent humid air	28.32±0.34	30.14±0.35	22 to 35	24 to 35
Direct evaporation 35 per cent humid air	Dried out. No larvæ.			
Direct evaporation 60 per cent humid air	32.21±0.71	34.48±0.90	16 to 47	26 to 49

* Temperature 23.5–25°C.

the escape of the air. The culture bottle, into which a stream of 35 per cent humid air was passed, dried up at the end of the 2nd day and no larvæ developed. The cultures into which 60 per cent air was admitted dried out rather rapidly, but not before many of the larvæ had pupated. Some of these emerged from pupation at the expected time but the majority was about 5 days late. The effect on the facet count is striking both as to the mean and the range. The upper range for the 23°C. stock counts is 39;[1] in this culture it has gone to 49, which is even above the upper range for 20°C. Unfortunately an atmometer test is impracticable, and no quantitative measure can be applied to this factor.

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The foregoing experiments are of value in showing that when a consistent procedure is followed and plenty of good moist food is present, the environment is practically constant except for variations in temperature and need not be taken into consideration in the interpretation of the bulk of breeding data now available for *Drosophila*.

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THE SIGNIFICANCE OF THE HYDROGEN ION CONCENTRATION FOR THE DIGESTION OF PROTEINS BY PEPSIN.

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One of the most striking peculiarities of enzyme action is the fact that the activity of the enzyme is limited to a definite range of acidity. If the solution is more or less acid than this the enzyme is practically inactive. Sörensen[1] showed that for a number of enzymes the determining factor was the hydrogen ion concentration and not the total acidity of the solution.

In attempting to account for this phenomenon it has usually been assumed that the influence of the hydrogen ion concentration was exerted upon the enzyme. Michaelis[2] pointed out, in the case of many enzymes, that if the activity of the enzyme was plotted against the hydrogen ion concentration of the solution the curve resembled strikingly that obtained when the ionization of a salt of a weak base and a strong acid was plotted in the same way. He concluded therefore that enzymes were weak bases or acids which formed salts with the acids or bases of the solution. These salts then dissociated into ions and the ions so formed were the active agents in the reaction. A similar explanation had already been proposed independently by Loeb[3] and by Nasse.[4] Michaelis' work rendered the hypothesis quite plausible. In the case of pepsin, however, it meets with several serious objections. In the first place, one of the strongest points of Michaelis' experiments was the fact that pepsin was found to have an isoelectric point at about pH 3.0 which agreed fairly well with the

[1] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

[2] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 58.

[3] Loeb, J., *Biochem. Z.*, 1909, xix, 534.

[4] Nasse, O., *Malys Jahrb.*, 1894, xxiv, 718.

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theory. Pekelharing and Ringer,[5] however, showed that in solutions of pure pepsin (prepared by Pekelharing's method from gastric juice) the pepsin was always negatively charged. This objection may of course be met by the statement that the pepsin under the actual conditions of hydrolysis (*i.e.* when in the protein solution) is not pure but is combined with some other substance and it is the ionization of this compound which determines the activity of the enzyme. An explanation similar to this has been offered by Michaelis.[6] The author[7] has shown, however, that pepsin combined with peptone or other decomposition products of the proteins is inactive and that it is only the free pepsin which takes part in the reaction. It was also found[8] that no positively charged pepsin could be found on the alkaline side of pH 3.3. Pepsin retains its activity up to pH 5, however, so that it seems unlikely that only positively charged pepsin is active, as assumed by Michaelis.

A second objection to Michaelis' view is the fact that the optimum hydrogen ion concentration for the activity of pepsin is found to vary with the substrate. This point has been emphasized by Long and Hull[9] (for trypsin) and by Ringer.[10] From Michaelis' point of view it is difficult to see how this can be. Neither of these objections, however, can in the author's opinion be considered as conclusive evidence against Michaelis' hypothesis. It could be stated for instance that pepsin contained several enzymes, one for each substrate and each with a different optimum. It seems simpler, however, to assume that the hydrogen ion concentration affects the condition of the substrate rather than the enzyme. This hypothesis has the advantage that it also accounts for the peculiar relation between the concen-

[5] Pekelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

[6] Michaelis, L., *Deutsch. med. Woch.*, 1920, xlvi, 1.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 468.

[9] Long, J. H., and Hull, M., *J. Am. Chem. Soc.*, 1917, xxxix, 1051. The same statement is made by Hedin and Hammerstein. The author has been unable to find the original work on which this statement is based. *Cf.* Hammerstein, O., and Hedin, S. G., A text-book of physiological chemistry, translated by Mandel, J. A., New York, 8th edition, 1915, 471. See also Abderhalden, E., and Fodor. A., *Fermentforsch.*, 1914–16, i, 591.

[10] Ringer, W. E., *Kolloid. Z.*, 1916, xix, 253.

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tration of the substrate and the rate of hydrolysis. Experiments described in a former paper[11] show that the rate of hydrolysis of protein solutions of varying concentration but the same pH was directly proportional to the amount of ionized protein present in the solution but not to the total concentration of protein. They agree therefore with the hypothesis that the ionized protein is the form which takes part in the reaction. If this explanation is correct it follows that the optimum hydrogen ion concentration for the activity of pepsin is also due to the increased ionization of the protein and must coincide with the hydrogen ion concentration at which the protein solution contains the greatest number of protein ions. (It was first suggested by Pauli[12] that the ionized protein was the form which was attacked. Euler[13] and Arrhenius[14] have made a similar suggestion. Ringer[15] considers also that the ionization of the substrate has an influence on the rate of digestion at least in the later stages.) It should be possible therefore to determine the optimum degree of acidity for pepsin digestion by measuring the conductivity of the protein solution. It will be shown below that this is true. It will further be shown that the range of hydrogen ion concentration in which the enzyme is active shifts in the same sense as the conductivity of the protein solution when a protein of different isoelectric point is used, and also that when the protein is insoluble the enzyme combines with it only over that range of hydrogen ion concentration in which the enzyme is active and in which the protein is ionized.

The Influence of the Isoelectric Point of the Protein on the Activity of Pepsin at Different Hydrogen Ion Concentrations.

Ringer[10] has already shown that the optimum hydrogen ion concentration for the digestion of proteins by pepsin varies somewhat with the protein hydrolyzed and with the acid used. He accounts for this phenomenon by the assumption that the hydration of the

[11] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.
[12] Pauli, W., *Arch. ges. Physiol.*, 1910, cxxxvi, 483.
[13] Euler, H., Allgemeine Chemie der Enzymes, Wiesbaden, 1910.
[14] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 44.
[15] Ringer, W. E., *Arch. Neerl. Physiol.*, 1917–18, ii, 571.

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protein determines the ease with which it is attacked by the enzyme. The viscosity is assumed to be a measure of the hydration. The same explanation has been proposed by Brücke,[16] by Pfliederer,[17] and recently by Traube.[18] The writer has been able to show,[19] however, that gelatin digests at the same rate in sulfuric or hydrochloric acid solution (provided the pH is the same) although the swelling, which Ringer considers a measure of the hydration, is much greater in hydrochloric than in sulfuric acid. It was also found[11] that the rate of digestion of egg albumin solutions decreased as the viscosity increased with the age of the solution instead of increasing as would be expected if the rate of digestion was determined by the hydration of the protein (as shown by the viscosity). Loeb[20] has shown that the ionization of the protein and the viscosity and swelling are all approximately proportional for a small range of acidity to the acid side of the isoelectric point. The maximum for the swelling and viscosity, however, occurs at about pH 3.4 whereas that for the ionization is much further to the acid side and agrees very well for that of the rate of digestion. This question will be discussed more fully below. It is clear, however, that in certain cases the swelling or viscosity and the ionization and rate of digestion may all be proportional. It would seem from the experiments described here that the determining factor for the rate of digestion is the ionization of the protein, and the swelling and viscosity are secondary characteristics which are probably also connected with the ionization.

It is known that, with most proteins, pepsin becomes inactive at a pH of about 4.5. This cannot be ascribed to the destruction of the enzyme since the author[8] found pepsin to be more stable in this range of acidity than at any other. The ionization of most proteins is very slight at this pH, however, so that it would be expected (from the hypothesis that it is the protein ion which is attacked by the enzyme) that little or no hydrolysis should occur at this point. Oxy-

[16] Brücke, E., *Sitzungsb. k. Akad. Wissensch. Math-naturw. Cl., Wien.*, 1859, xxxvii, 131.

[17] Pfliederer, R., *Arch. ges. Physiol.*, 1897, lxvi, 605.

[18] Traube, M., *Deutsch. med. Woch.*, 1919, xxvii,

[19] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[20] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39; 1920–21, iii, 85.

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hemoglobin, however, is isoelectric at a pH of about 6.8 (Michaelis[2]) so that it must be quite largely present as a salt and therefore ionized at a pH of 4.5. It would be predicted then, according to the hypothesis that the amount of protein ions present determines the rate of digestion of the protein, that hemoglobin should be digested by pepsin at pH 4.5 more rapidly than is egg albumin or gelatin at the same pH.

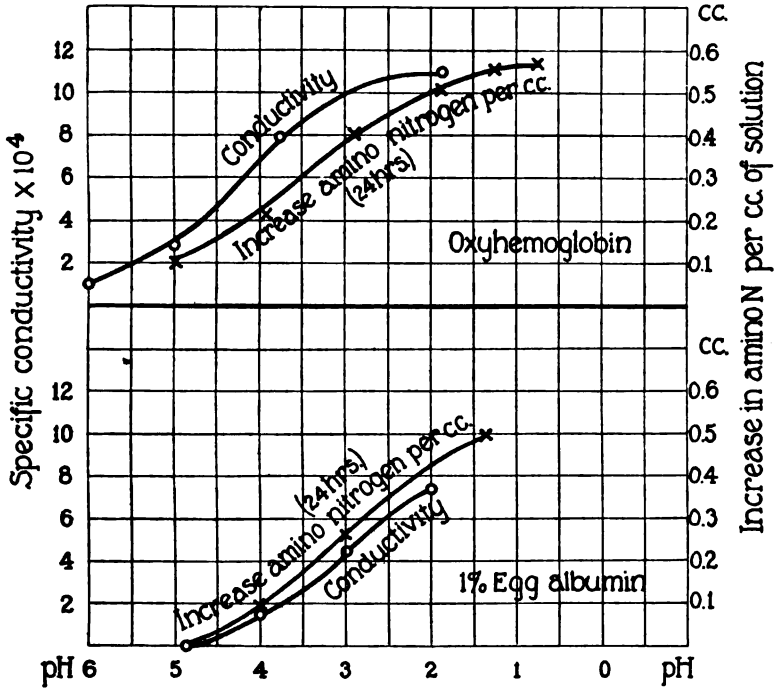

FIG. 1. Influence of pH on conductivity and rate of digestion of egg albumin and oxyhemoglobin solutions.

In order to test this prediction, parallel experiments were made to determine the rate of digestion and the conductivity of hemoglobin and egg albumin solutions at various hydrogen ion concentrations. The results of such an experiment are shown graphically in Fig. 1. It is clear that the conductivity and digestion curves, for each protein, as plotted against the pH of the solution are approximately parallel

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and also that the curves for the digestion and conductivity of the hemoglobin fall further to the left (*i.e.* to the alkaline side) than do the curves for the egg albumin.

The experiments cannot be considered as showing quantitative agreement between the rate of digestion and the conductivity of the solution since the digestion curve is given as the amount of protein decomposed in a certain time—a quantity which is not connected in any simple way with the rate of digestion. They are further complicated by the fact that the digestion in the region of the optimum acidity represents approximately 50 per cent of the complete digestion of the protein and therefore probably includes the secondary splitting of some of the primary products of the hydrolysis, and not purely the action on the protein itself. The conductivity on the other hand was measured on the protein solution itself. It is not possible to carry the digestion curve much beyond pH 5.0 owing to the rapid destruction of the enzyme.

EXPERIMENTAL.

Egg Albumin.—The egg albumin was crystallized three times as described by Hopkins and Pinkus[21] and then dialyzed under pressure of about 150 cm. of water at pH 4.8 until the specific conductivity of the solution was less than 1×10^{-4} reciprocal ohms. The solution was then diluted to 2 per cent with water. Increasing amounts of HCl were added to a series of 50 cc. portions of this solution and the total volume made up to 100 cc. 1 cc. of 2 per cent pepsin was then added to 25 cc. of these solutions and placed at 25°C. 1 cc. of the solution was analyzed by the Van Slyke[22] method for amino nitrogen after 0, 8, 24, and 36 hours. The curve given is the increase in cubic centimeters of amino nitrogen per cubic centimeter of solution after 24 hours. The 8 and 36 hour curves were similar.

Conductivity.—1 cc. of inactivated pepsin was added to another 25 cc. portion of the above solutions and the conductivity and pH of the solution were measured at 25°C. The conductivity of the egg albumin salt was determined from the conductivity of the solution by subtracting from the observed conductivity the conductivity of HCl of the same pH (Northrop[11]).

Oxyhemoglobin.—Erythrocytes from fresh defibrinated ox blood were washed with 7.8 per cent glucose solution until the conductivity of the suspension was less than 1×10^{-4} reciprocal ohms. The cells were then laked with ether, separated from the excess ether, and the ether in the solution removed *in vacuo*. The solution was then diluted to contain about 1 cc. of amino nitrogen per cubic centimeter as determined by the Van Slyke method. The conductivity of this

[21] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

[22] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

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solution was about 1×10^{-5} reciprocal ohms. Increasing amounts of HCl were added to 50 cc. portions of this solution and the total volume made up to 100 cc. The conductivity and digestion of the solution were then determined as described for the egg albumin.

The Optimum Hydrogen Ion Concentration for Pepsin Digestion.

The optimum hydrogen ion concentration for the activity of pepsin has been determined many times. All the methods used for following the digestion, however, have depended on the change in some physical property of the protein. It seemed of interest therefore to determine the optimum degree of acidity for the reaction when the hydrolysis was followed by means of the increase in amino nitrogen, which

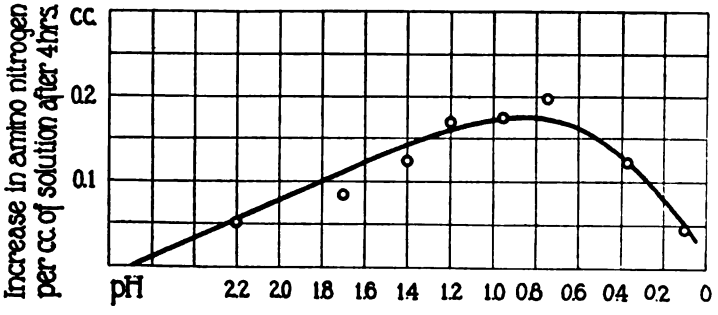

FIG. 2. Influence of pH on the rate of digestion of egg albumin.

probably represents correctly the actual course of the digestion. The method has the disadvantage, however, that only comparatively large changes can be followed. The results of an experiment made with egg albumin solutions of different pH (adjusted with HCl) are given in Fig. 2.

The time of digestion was 4 hours. The figure shows that the optimum acidity for the digestion as determined by the increase in amino nitrogen is at about pH 1.0 (0.1 N). This is slightly more acid than that found by Sörensen,[1] Michaelis and Mendelssohn,[23] or Okada,[24] and much more acid than that found by Ringer.[15] It must be remembered,

[23] Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxv, 1.
[24] Okada, S., *Biochem. J.*, 1916, x, 126.

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however, that the chemical changes followed by the increase in amino nitrogen represent much more complete hydrolysis than those followed by the other authors. The curve therefore probably does not represent the correct optimum for the digestion of the protein itself but probably also the digestion of some of the primary products. The careful work of Ringer has shown that the optimum zone for the digestion of these products extends further to the acid side than the zone for the digestion of the protein itself. This probably accounts for the difference in the optimum found by the different methods and agrees with the results of Sörensen[1] who found that the optimum shifts to the acid side with more complete digestion.

The Effect of Adding Salt with a Common Ion to a Solution Already Containing the Optimum Amount of Acid.

It will be noted from the curve (Fig. 2) that the amount of digestion increases with increasing amounts of acid in the solution until the hydrogen ion concentration is about 0.1 N and then decreases. According to the hypothesis that it is the ionized protein which is hydrolyzed by the pepsin, the increase in digestion from pH 4.0 to 1.0 is due to the fact that as acid is added to the albumin more protein salt and hence more protein ions are formed in the solution, until all the albumin is present as salt. The addition of a further amount of acid serves to depress the concentration of protein ions again due to the effect of the common ion. According to this mechanism the hydrogen ion concentration is the determining factor on the alkaline side of the optimum while on the acid side the concentration of the anion is the determining factor. It can be predicted therefore that if a solution of a salt (having the same anion as the acid) is added to a solution of the protein which already contains the optimum amount of acid, the depressing effect of the salt on the digestion should be the same as if excess acid had been added, provided the final anion concentration is the same. The conductivity of the albumin salt should also be diminished. In the case of egg albumin this cannot be experimentally verified owing to the fact that the albumin precipitates under these conditions, and also since the conductivity of the protein in such strongly acid solutions is so small, compared to

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the total conductivity, as to render the measurement very uncertain. It will be shown later, however, that in the case of gelatin the decrease in conductivity can be followed and is proportional to the decrease in the rate of digestion.

TABLE I.

Increase in Amino Nitrogen per Cc. of Solution Containing Normal Total Chlorine Concentration Furnished by Different Salts.

Original solution 0.5 N HCl.
Made up to 1.0 N chloride concentration with salt noted.

Salt.	pH	Increase in NH_2 nitrogen per cc. after 6 hrs. at 25°C.
		cc.
0	0.42	0.25
		0.26
NaCl	0.40	0.15
		0.13
KCl	0.42	0.15
		0.15
$CaCl_2$	0.41	0.17
		0.15
$MgCl_2$	0.42	0.12
		0.11
$SrCl_2$	0.42	0.17
		0.18
$AlCl_3$	0.40	0.10
		0.11
HCl	0.13	0.13
		0.14

Table I contains a summary of the results of such an experiment in which a series of egg albumin solutions all containing a total chlorine ion concentration of 0.5 N and at a pH of 0.42 were brought to a total chlorine ion concentration of 1.0 N by the addition of the salts noted or excess acid. The final solutions therefore were all

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1.0 N in respect to the chlorine ion but those which had been brought to this concentration by the addition of salts were of course much less acid than the one to which excess acid had been added. The amount of digestion in all the solutions containing the same chlorine ion concentration was approximately the same, however. This result indicates that the controlling factor on the acid side of the optimum is the anion concentration and not the hydrogen ion concentration. As a corollary of this it can be stated that the addition of salt to a protein solution will cause the optimum hydrogen ion concentration for digestion to be shifted to the alkaline side. This was the effect noted by Michaelis and Mendelssohn.[23]

The above question has recently been examined by Gyemant.[25] This author found, however, the optimum pH for digestion remained at about pH 2.0 even though the anion concentration was the same in all the solutions. He concludes therefore that the decrease in the rate of digestion on the acid side of the optimum is due to the influence of the hydrogen ion on the pepsin as proposed by Michaelis.

The experiments described in this paper are complicated by the fact that the egg albumin was partially precipitated by the high concentrations of salt and acid used. This may account for the difference between the present results and those of Gyemant. The discrepancy may also be due to the fact that Gyemant followed the reaction by means of the increase in non-protein nitrogen whereas the author used the increase in amino nitrogen. In view of Gyemant's results and of the complicating factor of precipitation in the present experiments, they cannot be considered as conclusive evidence in favor of the view that the anion alone affects the digestion on the acid side of the optimum. It is possible that both ions are active. It appears to the author, however, that the action is exerted on the protein rather than the enzyme in view of the fact that different proteins show slightly different optimum pH, and of the close connection between the conductivity and rate of digestion of gelatin solutions (as described below in this paper).

The Conductivity and Rate of Digestion of Gelatin Solutions.

It was mentioned above that determinations of the conductivity of egg albumin solutions in strongly acid solution were made uncertain owing to the precipitation of the protein. This difficulty is not encountered with gelatin. Gelatin possesses the further advantage that the rate of digestion in the very early stages may be easily fol-

[25] Gyemant, A., *Biochem. Z.*, 1920, cv, 155.

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lowed by noting the time necessary to cause a certain degree of liquefaction of the gelatin.

A series of gelatin solutions, containing 5 per cent dry weight of gelatin and adjusted to various hydrogen ion concentrations by means of HCl, were prepared. The gelatin had previously been purified as described by Loeb.[26] The conductivity of the solutions and the time necessary for them to reach an easily determined degree of liquefaction were then determined. The reciprocal of this time is plotted in the curve as the rate. Fig. 3 and Table II show the result of a typical experiment of this kind. It is clear that the rate of diges-

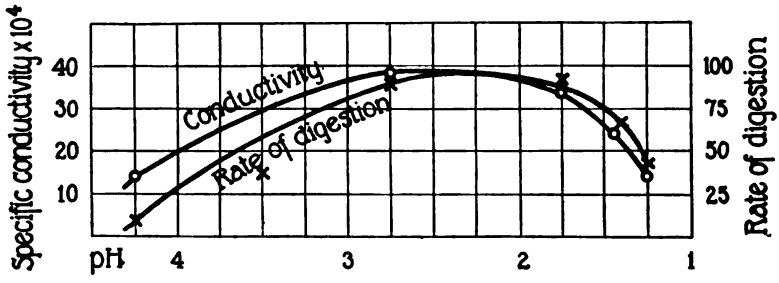

FIG. 3. Influence of pH on the rate of digestion and conductivity of gelatin solutions.

tion and the conductivity of the solution have their maximum value at the same hydrogen ion concentration, and that the curves are nearly parallel throughout. The rate of digestion decreases slightly more rapidly than the conductivity of the solution on the alkaline side of the optimum and slightly less rapidly on the acid side. This peculiarity was noted in all the experiments made and can hardly be ascribed to experimental errors. It shows that the digestion on the alkaline side of the optimum is slightly less rapid than would be predicted from the conductivity data and that it is slightly more rapid on the acid side. The divergence on the acid side is due to the fact that in such strongly acid solutions the acid itself has some action on the protein as was shown by control experiments without any pepsin. The correction is too uncertain to be applied to the figures, however.

[26] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

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It can only be said that such a correction is necessary and that it would be in the right sense. The divergence on the alkaline side is probably due to the fact that the amount of hydrolysis selected as the end-point represented too great a percentage change in the original substrate concentration to assume that the substrate concentration remained constant during the course of the experiment.

TABLE II.

pH, Conductivity, and Rate of Digestion of Gelatin Solutions.

Gelatin, 5 per cent dry weight in solution of total (approximate) concentration of HCl noted. Temperature, 37°C.

Approximate total concentration of HCl.	pH	$C_H \times 10^4$	Specific conductivity of (Reciprocal ohms × 10^4.)			Time for gelatin to liquefy.	
			Solution.	HCl of same pH as solution.	Gelatin chloride (= κ solution − κHCl).	Hours.	Rate = $\frac{40}{\text{hrs.}}$
N							
0.02	4.23	0.60	17.2	0.27	17.0	4.5	9
0.04	3.50	3.16	33.1	1.47	31.6	1.1	36
0.06	2.78	16.6	48.2	7.7	40.5	0.40	100
0.08	1.78	166.0	110.0	76.0	34.0	0.42	95
0.10	1.48	331.0	175.0	151.0	24.0	0.65	62
0.12	1.26	550.0	260.0	245.0	15.0	0.92	43

EXPERIMENTAL.

50 gm. (dry weight) of purified isoelectric gelatin were dissolved in warm water and the volume was made up to 500 cc. Increasing amounts of HCl were then added to a series of 50 cc. portions of this solution and the volume of each portion was then made up to 100 cc. 2 cc. of 2 per cent pepsin solution were then added to 75 cc. of each of the above solutions and the solutions put in the water bath at 37°C. At short intervals 5 cc. samples were pipetted from each of the solutions into a series of test-tubes containing 2 cc. of water. These tubes were then placed in a water bath at 2°C. for 10 minutes, taken out, and the degree of liquefaction was compared with that of a standard tube. (This is a slight modification of the method of Fermi as described by Dernby.[27]) This procedure was repeated until a sample from each of the tubes showed the same degree of liquefaction as the standard tube. In this way the time necessary to produce a certain degree of liquefaction can be accurately and easily determined. The pH and con-

[27] Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

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ductivity of the solution were determined on the remaining 25 cc. of solution to which had been added the equivalent amount of inactivated pepsin. The determinations were made as described above except that the measurements were made at 37°C.

The Combination of Pepsin and Gelatin.

In a former paper[28] it was shown that the amount of pepsin which combined with a given quantity of coagulated egg albumin depended entirely on the reaction of the solution in which the egg albumin was suspended. The greatest amount of pepsin was combined when the

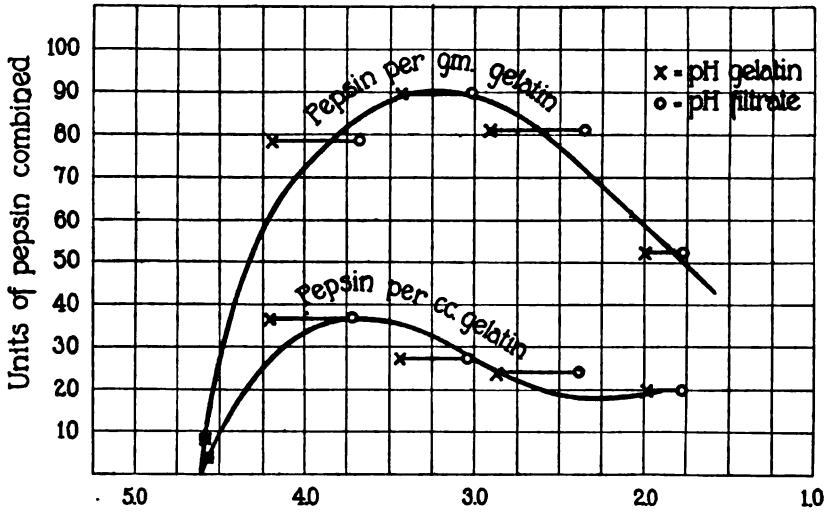

FIG. 4. Influence of pH on the combination of pepsin and gelatin.

solution had a reaction of pH 2.5 to 3.0. It was pointed out that this was probably part of the mechanism that caused insoluble proteins to digest more rapidly at this reaction than at any other since it seems that the rate of digestion must depend on the amount of pepsin in the solid protein.

These experiments have been repeated with gelatin and show in general the same result. The results of such an experiment are given in Fig. 4 and Table III. The figures show that a greater amount

[28] Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 113.

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of gelatin and pepsin is combined at about pH 3.0 than in either more or less acid solutions. In the case of gelatin the volume varies greatly with the reaction owing to the effect of the acid on the swelling of gelatin. The swelling is greatest at about pH 3.4 (*cf.* Loeb[20]). It might be supposed therefore that more pepsin was combined with the gelatin at about this degree of acidity simply because there was

TABLE III.

Combination of Gelatin and Pepsin.

5 gm. of isoelectric purified gelatin (= 0.75 gm. of dry weight) suspended in 200 cc. of HCl of strength noted and left 16 hours at 2°C. Filtered and washed twice with 100 cc. of water (5°C.) and total volume made up to 75 cc. 5 cc. of 2 per cent pepsin added. Allowed to stand 20 min. at 5° with occasional stirring. 4 cc. of supernatant fluid pipetted off and pepsin determined* in 1 cc. of this sample. Gelatin filtered and volume of filtrate measured. Gelatin melted and pH determined of this and of the filtrate.

Concentration of HCl.	pH of		Volume of filtrate.	Volume of gelatin (= 80 − volume of filtrate).	Units of pepsin.			
	Filtrate.	Gelatin.			Pepsin per cc. of filtrate.	Total pepsin in filtrate (*a*).	Total pepsin in gelatin (115–*a*).	Pepsin per 10 cc. of gelatin.
			cc.	*cc.*				
0	5.3	4.6	63	17	1.7	107.0	8	5
$\frac{M}{256}$	3.6	4.2	57	23	0.65	37.0	78	34
$\frac{M}{64}$	3.0	3.4	47	33	0.52	25.0	90	27
$\frac{M}{8}$	2.4	2.9	47	33	0.71	33.0	82	25
$\frac{M}{4}$	1.8	2.0	53	27	1.17	62.0	53	20
0 Control. No gelatin.	5.2		80	0	1.44	115.0	0	0

* *Cf.* Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113. The relative amount of pepsin is taken as the reciprocal of the time in hrs. required to cause a 5 per cent change in the conductivity of a 5 per cent egg albumin solution titrated to pH 1.7 with hydrochloric acid when 1 cc. of pepsin solution is added to 25 cc. of egg albumin at 37°C. The unit of pepsin is taken as that amount which when dissolved in 1 cc. and added to 25 cc. of the egg albumin solution will cause a change of 5 per cent in the conductivity in 1 hr.

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a greater volume of gelatin present at this point. It will be seen, however, from Table II and Fig. 3 that this is not true since the figures show that there is a maximum even when the results are calculated to the basis of pepsin per cubic centimeter of gelatin. That is, there is not only more pepsin combined with a gram of gelatin at this pH but also the concentration of the pepsin in the gelatin is greatest here. There is considerable uncertainty as to the pH measurements since, as the table shows, the reaction of the liquid was always considerably more acid than that of the gelatin. In most of the experiments the difference was much more marked than in the experiment given; in some cases the maximum fell at about pH 2.2. This agrees much more closely with the optimum acidity found for digestion and for the ionization of the protein. Owing to the uncertainty of the pH measurement, however, it is probably better to make no definite statement as to the exact position of the optimum acidity for the combination between the gelatin and pepsin. The determining factor in regulating the amount of pepsin which is combined with the gelatin is the chemical condition of the gelatin and pepsin and not a difference in the rate of diffusion of the pepsin since the same curve is obtained irrespective of the time (after the first few minutes) during which the gelatin is left in the solution. The simplest explanation would seem to be that the gelatin combines only with the ionized protein and the amount combined therefore is dependent on the amount of ionized gelatin present. Pepsin therefore behaves just as do the inorganic anions studied by Loeb[20] as far as the influence of the hydrogen ion concentration on the combination is concerned.

DISCUSSION AND SUMMARY.

The experiments described above show that the rate of digestion and the conductivity of protein solutions are very closely parallel. If the isoelectric point of a protein is at a lower hydrogen ion concentration than that of another, the conductivity and also the rate of digestion of the first protein extends further to the alkaline side. The optimum hydrogen ion concentration for the rate of digestion and the degree of ionization (conductivity) of gelatin solutions is the same, and the curves for the ionization and rate of digestion as

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plotted against the pH are nearly parallel throughout. The addition of a salt with the same anion as the acid to a solution of protein already containing the optimum amount of the acid has the same depressing effect on the digestion as has the addition of the equivalent amount of acid. These facts are in quantitative agreement with the hypothesis that the determining factor in the digestion of proteins by pepsin is the amount of ionized protein present in the solution. It was shown in a previous paper[11] that this would also account for the peculiar relation between the rate of digestion and the concentration of protein. The amount of ionized protein in the solution depends on the amount of salt formed between the protein (a weak base) and the acid. This quantity, in turn, according to the hydrolysis theory of the salts of weak bases and strong acids, is a function of the hydrogen ion concentration, up to the point at which all the protein is combined with the acid as a salt. This point is the optimum hydrogen ion concentration for digestion, since the solution now contains the maximum concentration of protein ions. The hydrogen ion concentration in this range therefore is merely a convenient indicator of the amount of ionized protein present in the solution and takes no active part in the hydrolysis. After sufficient acid has been added to combine with all the protein, *i.e.* at pH of about 2.0, the further addition of acid serves to depress the ionization of the protein salt by increasing the concentration of the common anion. The hydrogen ion concentration is, therefore, no longer an indicator of the amount of ionized protein present, since this quantity is now determined by the anion concentration. Hence on the acid side of the optimum the addition of the same concentration of anion should have the same influence on the rate of digestion irrespective of whether it is combined with hydrogen or some other ion (provided, of course, that there is no other secondary effect of the other ion). The proposed mechanism is very similar to that suggested by Stieglitz and his coworkers[29] for the hydrolysis of the imido esters.

Pekelharing and Ringer[5] have shown that pure pepsin in acid solution is always negatively charged; *i.e.*, it is an anion. The experiments described above show further that it behaves just as would be expected of any anion in the presence of a salt containing the protein ion as the cation and as has been shown by Loeb[20] to be the case with inorganic anions.

[29] Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 164, 402, 437, 586, 719.

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Nothing has been said in regard to the quantitative agreement between the increasing amounts of ionized protein found in the solution (as shown by the conductivity values) and the amount predicted by the hydrolysis theory of the formation of salts of weak bases and strong acids. There is little doubt that the values are in qualitative agreement with such a theory. In order to make a quantitative comparison, however, it would be necessary to know the ionization constant of the protein and of the protein salt and also the number of hydroxyl (or amino) groups in the protein molecule as well as the molecular weight of the protein. Since these values are not known with any degree of certainty there appears to be no value at present in attempting to apply the hydrolysis equations to the data obtained.

It it clear that the hypothesis as outlined above for the hydrolysis of proteins by pepsin cannot be extended directly to enzymes in general, since in many cases the substrate is not known to exist in an ionized condition at all. It is possible, however, that ionization is really present or that the equilibrium instead of being ionic is between two tautomeric forms of the substrate, only one of which is attacked by the enzyme. Furthermore, it is clear that even in the case of proteins there are difficulties in the way since the pepsin obtained from young animals, or a similar enzyme preparation from yeast or other microorganisms, is said to have a different optimum hydrogen ion concentration than that found for the pepsin used in these experiments. The activity of these enzyme preparations therefore would not be found to depend on the ionization of the protein. It is possible of course that the enzyme preparations mentioned may contain several proteolytic enzymes and that the action observed is a combination of the action of several enzymes. Dernby[27] has shown that this is a very probable explanation of the action of the autolytic enzymes. The optimum hydrogen ion concentration for the activity of the pepsin used in these experiments agrees very closely with that found by Ringer for pepsin prepared by him directly from gastric juice and very carefully purified. Ringer's pepsin probably represents as pure an enzyme preparation as it is possible to prepare. There is every reason to suppose therefore that the enzyme used in this work was not a mixture of several enzymes.

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RADIOACTIVITY AND PHYSIOLOGICAL ACTION OF POTASSIUM.

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I. INTRODUCTION.

The cations Na, K, and Ca are essential constituents of physiologically balanced salt solutions such as blood serum, tissue fluids, and sea water, and, in the absence of one of these ions, physiological processes cannot, as a rule, occur for any great period of time. Zwaardemaker[1] has recently advanced the interesting idea that the indispensability of potassium in cardiac action is due to the slight radioactivity of that element. To prove this idea, he has demonstrated that other radioactive substances, *e.g.* thorium, uranium, ionium, radium, etc., can replace the K ion in restoring heart beat after the heart has stopped beating in a Ringer solution containing no potassium. The most significant fact demonstrated by Zwaardemaker and his collaborators is that for the replacement of potassium by other radioactive substances, equiradioactive doses are required. Zwaardemaker's conclusion that the action of potassium in physiologically balanced salt solutions is a result of its radioactivity is of such great importance that it seemed justifiable to test the applicability of this view to physiological processes other than the heart beat.

As a result of experiments by Herbst[2] and others, it is known that sea urchin eggs are unable to develop when placed in potassium-free sea water immediately after fertilization, and Herbst found that rubidium and cesium could replace potassium to a limited extent. It seemed to us that a further study of the replacement of K by cesium

[1] Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273.

[2] Herbst, C., *Arch. Entwcklngsmechn. Organ.*, 1901, xi, 617.

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and certain radioactive substances might help in deciding definitely whether or not the physiological influence of potassium is dependent on its radioactive properties.

II. EXPERIMENTAL.

The method used in this work was as follows. Eggs of *Arbacia* were fertilized in potassium-free artificial sea water of the following composition: 100 cc. of M/2 NaCl + 7.8 cc. of M/2 $MgCl_2$ + 3.8 cc. of M/2 $MgSO_4$ + 1.75 cc. of M/2 $CaCl_2$. To this solution enough M/10 $NaHCO_3$ (usually 0.2 cc. per 25 cc. of solution) was added to bring the pH to between 7.4 and 8.0. The chemicals used were Kahlbaum's purest preparations. Both glass- and metal-distilled water were used in preparing the solutions. After fertilization, the eggs were washed three times in similar K-free artificial sea water and were then placed in various solutions to observe development. In all experiments, some of the fertilized eggs were placed in (*a*) normal sea water, and (*b*) alkaline K-free sea water for control. In no case where eggs were placed in K-free sea water did the development go beyond the sixteen cell stage, and in a few hours the eggs were disintegrated. All sea water controls developed into normal plutei.

The criterion used in these experiments for the adequacy of the substitutes for potassium was the formation of normal swimming blastulæ within 24 hours. In reality development went beyond this stage in most instances.

A. Potassium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of K-free sea water to which was added enough KCl to make the total concentration of KCl in the dishes M/4,600, M/2,300, M/1,300, M/850, M/660, M/550, M/470, M/370, M/330, etc., up to M/18, the latter concentration of KCl being about 5.5 times that present in normal sea water. The pH in these and succeeding experiments was about 7.4.

From Table I we see that fertilized eggs do not form swimming larvæ in artificial sea water with a concentration of KCl lower than M/660. We also see that a certain excess of potassium, *i.e.* 5.5 times its normal concentration in sea water, does not interfere with development up to the gastrula stage.

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B. Rubidium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of K-free sea water to which was added enough RbCl to make the total concentration of RbCl in the dishes M/4,600, M/2,300, M/1,300, M/850, M/660, M/550, M/470, M/370, M/330, etc., to M/18.

The experiments proved that RbCl is entirely able to replace KCl in the development of swimming blastulæ and that the minimal concentration of RbCl needed is M/660, approximately that needed when KCl is used.

TABLE I.

Concentration of Potassium Needed for Development.

Molecular concentration of KCl.	Stage of development after	
	5 hrs.	24 hrs.
0	2–16 cells disintegrating.	Dead.
M/4,600	2–16 " "	"
M/2,300	2–16 " "	"
M/1,300	Few as far as 64 cells, mostly disintegrating.	"
M/850	" " " " 64 " " "	"
M/660	" " " " 64 " " "	Few swimming gastrulæ.
M/550	Some disintegrating, many good early blastulæ.	Swimming gastrulæ.
M/470	Almost all early blastulæ.	All gastrulæ.
M/370	" " " "	" "
M/330	All early blastulæ.	" "
M/150	" " "	" "
M/125	" " "	" "
M/100	" " "	" "
M/52	" " "	" "
M/27	" " "	" "
M/18	" " "	" "

C. Cesium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of potassium-free sea water to which was added enough CsCl to make the total concentration of CsCl in the dishes M/4,600, M/2,300, M/1,300, M/1,000, M/850, M/660, M/500, M/250, M/125, etc., to M/18.

From Table II we see that CsCl can replace potassium chloride and that the minimal molecular concentration required for this purpose is practically identical for both salts. If the action of potassium is

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due to its radioactivity, we should have to conclude that cesium has the same degree of radioactivity as potassium which is contrary to the facts known at present. Development with CsCl is possibly slower than with KCl, and the development does not go so far as in

TABLE II.

Concentration of Cesium Needed for Development.

Molecular concentration of CsCl.	Stage of development after	
	6 hrs.	24 hrs.
0	2–16 cells disintegrating.	Dead.
M/4,600	All disintegrating.	"
M/2,300	8–16 cells disintegrating.	"
M/1,300	8–16 " "	"
M/1,000	Few 16–32 cells, most disintegrating.	"
M/850	Few 64 cells, most disintegrating.	"
M/660	" 64 " " "	"
M/500	About 50 per cent in 64 cell stage.	About 50 per cent very slowly swimming blastulæ.
M/250	Most in 64 or 128 cell stage.	About 50 per cent slowly swimming blastulæ.
M/170	Practically all 128 cells.	Still more swimming blastulæ.
M/125	" " 128 "	Mostly rapidly swimming blastulæ; few gastrulæ.
M/100	" " 128 "	Most blastulæ; some rapidly swimming gastrulæ.
M/80	" " 128 "	Most blastulæ; some rapidly swimming gastrulæ.
M/64	" " 128 "	Same, but more gastrulæ.
M/44	" " 128 "	Swimming blastulæ, many degenerating.
M/33	" " 128 "	Mostly slowly swimming blastulæ.
M/18	" " 128 "	Mostly swimming gastrulæ.

the case of KCl probably on account of the greater toxicity of cesium which in rather low concentrations kills the larvæ.

D. Thorium Chloride Experiments.—It seemed important to determine whether or not potassium could be replaced by a radioactive element like thorium. Zwaardemaker found that in winter a solu-

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tion of M/10,000 and in summer about M/100,000 thorium nitrate could replace potassium in his experiments on the heart. In our experiments, we fertilized eggs as described and after thorough washing in K-free sea water they were placed in dishes containing 25 cc. of K-free sea water and enough thorium chloride to make the total concentration of $ThCl_4$ in the dishes M/200,000, M/100,000, M/66,000, M/33,000, M/25,000, M/21,000, M/11,000, M/7,500, M/4,800, M/3,300, and M/2,100. To these solutions, which were quite acid, enough M/10 $NaHCO_3$ was added to bring the pH between 7.2 and 8.0. In all these

TABLE III.

Effect of Thorium in Replacing Potassium.

Molecular concentration of $ThCl_4$	Stage of development after	
	4 hrs.	24 hrs.
0	2–8 cell stage disintegrating.	Dead.
M/200,000	2–8 " " "	"
M/100,000	2–8 " " "	"
M/66,000	2–8 " " "	"
M/33,000	2–8 " " "	"
M/25,000	2–8 " " "	"
M/21,000	2–8 " " "	"
M/11,000	2–8 " " "	"
M/7,500	2–8 " " "	"
M/4,800	2–8 " " "	"
M/3,300	2–8 " " "	"
M/2,100	2–8 " " "	"

cases the Th was probably in suspension and not in true solution and in the three highest concentrations of $ThCl_4$ addition of bicarbonate caused visible precipitation. Since Zwaardemaker's Ringer solutions were also slightly alkaline the Th in his solutions was also probably in suspension, but this would not interfere with the radioactivity since the radioactivity depends on the changes in the nucleus of an atom and since these nuclear changes do not depend on whether or not the substance is in true solution. As is seen in Table III in no case where Th was used to replace K did we observe the development of swimming blastulæ and in all dishes disintegration occurred before the sixteen cell stage was reached just as in the K-free sea water

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control. It was shown that death in the thorium experiments was due to lack of K and not to the toxicity of $ThCl_4$, because when $ThCl_4$ was added to normal sea water in the same concentrations in which it was used to replace KCl it in no way interfered with the formation of normal larvæ, owing probably to the fact that the Th was in suspension and not in true solution. The radioactivity of the $ThCl_4$ was not sufficient to interfere with the development of the eggs up to the pluteus stage at least.

E. Uranium Acetate Experiments.—A series of experiments with the radioactive element uranium also gave negative results; *i.e.*, in no case were swimming larvæ obtained where uranium acetate was used to replace potassium and the eggs died before the sixteen cell stage was reached. The concentrations of uranium acetate used in the K-free sea water were M/125,000, M/62,500, M/42,000, M/32,000, M/25,000, M/16,000, M/12,500, M/11,000, M/6,800, M/4,700, M/3,000, and M/2,100. The pH in these solutions was brought to about 7.6 through the addition of $NaHCO_3$ and in the two highest concentrations some uranium was visibly precipitated. The lack of development of the eggs here was not due to the toxicity of the uranium salt because, when the uranium acetate was added to sea water in the same concentrations, normal larvæ developed except in the two highest concentrations and here the pH was found to be below 7. The uranium as well as the thorium was probably in suspension in the sea water and not in true solution but this, of course, did not influence the radioactivity. It is of interest to note that, here and in the thorium experiments, the radioactive elements did not inhibit the action of K as Zwaardemaker seems to assume.

III. Theoretical Remarks.

It appears from the foregoing experiments that in the development of the fertilized egg of *Arbacia* up to the blastula-gastrula stage potassium can be replaced by the non-radioactive Cs ion, the minimum concentration required for development being practically the same for both cations (KCl minimum concentration = M/660, CsCl minimum concentration = M/500). Furthermore, it is apparent that K cannot be replaced in our experiments by suspensions of the radioactive elements thorium and uranium. Hence, the action of potassium in the

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development of the eggs of *Arbacia* cannot be attributed to its very slight radioactivity.

These results would seem at variance with the findings of Zwaardemaker and his coworkers in their experiments on the heart were it not for the fact that they also were able to replace potassium by cesium, which Zwaardemaker designates as "physiologically but not physically" radioactive. As a matter of fact nobody has thus far shown that Cs possesses more radioactivity than any other non-radioactive element; *e.g.*, Na. Zwaardemaker's observation that a heart which has ceased to beat in a solution lacking KCl can be resuscitated by radioactive substances may be explained without the assumption that K acts through its radioactivity. Lingle[3] found that when the ventricle of the heart of the turtle was suspended in a moist chamber where the air was replaced by pure oxygen the strip was able to beat for a considerable time, as long as 3 days, after the beats had been initiated by submersing the ventricle for a short time in a pure NaCl solution. The beats continued in some cases until the putrefaction of the heart tissues put a stop to them. This experiment proves that the KCl is not needed to provide the stimulus for the heart beat but that it is only needed to counteract some of the toxic effects of a pure NaCl solution when the heart is submersed in such a solution. Lingle found, moreover, that the heart which had stopped beating in a pure solution of NaCl began to beat again when 10 cc. of 3 per cent H_2O_2 were added to 90 per cent of the isotonic solution of NaCl. He could show by control experiments that only the bubbles of O_2 with which the muscle became frosted were responsible for the resuscitation. The ventricle thus resuscitated could then continue to beat for many hours. Lingle suggests that the oxygen supply in a solution is inadequate for a heart beating in a pure solution of NaCl unless a more rapid source of supplying oxygen than mere diffusion from the air is provided. We do not know how the increased supply of oxygen can resuscitate the heart which had ceased to beat in a pure solution of NaCl but the tentative suggestion may be permitted that this effect is due to the transformation of a harmful substance formed during the activity of the heart in the absence of K, or Ca, or

[3] Lingle, D. J., *Am. J. Physiol.*, 1902–03, viii, 75.

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both, into a less harmful one through a more rapid oxidation. Lind has shown that penetrating rays can cause the formation of water from O and H and that H_2O_2 seems to be an intermediate product in the reaction.[4] On the basis of these observations it is quite probable that H_2O_2 is formed or that oxygen is activated in some other form when penetrating rays go through the cells of the heart, and that the rate of oxidation is increased in the cells of the heart. This might explain Zwaardemaker's experiments on the restoration of the heart beat by U, Th, and radium without compelling us to assume that KCl, Rb, and even Cs act physiologically by radioactivity.

CONCLUSIONS.

1. The non-radioactive cesium ion can replace the potassium ion almost quantitatively in solutions required for the development of the egg of the sea urchin into swimming blastulæ.

2. Thorium chloride and uranium acetate cannot replace the potassium chloride in the solutions required for the development of the egg.

3. Thorium chloride and uranium acetate do not antagonize the action of the potassium contained in sea water upon the development of eggs.

[4] Lind, S. C., *J. Am. Chem. Soc.*, 1919, xli, 551.

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CHEMICAL CHARACTER AND PHYSIOLOGICAL ACTION OF THE POTASSIUM ION.

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I. INTRODUCTION.

Zwaardemaker[1] has recently made the interesting suggestion that the rôle of potassium in physiologically balanced salt solutions—*e.g.* the blood or the sea water—is due to the very slight radioactivity of this element and not to its chemical character as determined by its position in the series of elements. It has been pointed out by R. F. Loeb[2] in another paper in this *Journal* that K cannot be replaced by Th and U as far as the development of the sea urchin egg is concerned, that the non-radioactive element Cs is capable of replacing potassium to some extent in this case, and that Zwaardemaker's observations on the influence of radioactive substances on the heart beat might be explained without the assumption that the physiological action of K is due to its radioactivity.

This then suggests that the physiological action of potassium is due to its chemical character. We know through the work of Sir Ernest Rutherford that radioactivity is caused by an explosive charge in the nucleus of the atom while the chemical and most of the physical properties of the atom depend upon its external ring or shell of electrons. These latter properties are repeated periodically in the series of elements arranged by their atomic numbers and if we can show that the physiological action of an element corresponds to its position in the periodic table we know that we are dealing with purely chemical effects and not with radioactive effects. We intend to show in an indirect way that the action of K in physiologically balanced salt

[1] Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273

[2] Loeb, R. F., *J. Gen. Physiol.*, 1920–21, iii, 229.

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solutions corresponds to its purely chemical character; *i.e.*, its position in the periodic table (or rather to its atomic number and arrangement of external electrons).

II. The Resemblance in the Antagonistic Effects of the NH_4 and K Ions.

According to their physiological action the ions of the alkaline metals can be arranged in two distinct groups, the one including Li and Na, the other K, Rb, and Cs. The difference in the two groups is noticeable in various phenomena. Isotonic solutions of LiCl and NaCl will give rise to muscular twitchings while KCl, RbCl, and probably CsCl will not. Experiments on the egg of *Fundulus* show that toxic solutions of salts with bivalent metals, such as $MgCl_2$ or $CaCl_2$, can be rendered less toxic by the addition of KCl, RbCl, or CsCl, but not or practically not by the addition of NaCl or LiCl.[3] It is known that the NH_4 ion resembles in its chemical behavior the K ion more closely than it does the sodium ion, and Langmuir[4] has utilized this fact in support of his theory of the cubical atom. If it be true that the physiological action of K depends upon its chemical character, the close resemblance between the chemical character of NH_4 and K should express itself in phenomena of antagonism. NH_4Cl is generally very toxic for cells but it can be used with good effect in experiments on the egg of *Fundulus* which is surrounded by a rather impermeable membrane. In experiments on *Fundulus* it can be shown that the antagonistic action of the NH_4 ion is like that of the members of the K group and not like that of the members of the Na group.

When newly fertilized eggs of *Fundulus* are put into a 5 M/32 solution of $CaCl_2$ practically no egg (*i.e.* less than 2 per cent of the eggs) can form an embryo. When the 5 M/32 $CaCl_2$ solution is made up in solutions of different chlorides instead of in H_2O it is found that in LiCl and NaCl the toxicity of the $CaCl_2$ solution is not diminished. When, however, the 5 M/32 $CaCl_2$ solution is made up in KCl, RbCl, CsCl, or NH_4Cl a considerable percentage of the eggs can develop into embryos as is shown in Table I.

[3] Loeb, J., *J. Biol. Chem.*, 1914, xix, 431.
[4] Langmuir, I., *J. Am. Chem. Soc.*, 1920, xlii, 274.

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The same fact can be demonstrated equally well with other toxic solutions; *e.g.*, Na_3 citrate. If newly fertilized eggs of *Fundulus* are put into M/100 Na_3 citrate practically no egg can form an embryo, and the result remains the same if the M/100 solution of Na_3 citrate is made up in different concentrations of LiCl or NaCl. When, however, the M/100 solution of Na_3 citrate is made up in KCl, RbCl, CsCl, or NH_4Cl a considerable number of eggs develop into embryos as indicated in Table II.

TABLE I.

	Percentage of newly fertilised *Fundulus* eggs which can form embryos in 5 M/32 $CaCl_2$ when this solution is made up in						
	0	M/80	M/40	M/20	M/10	M/5	3 M/10
LiC.....	1.5	0	0	0	0	0	0
NaCl.....		1	2	1	0	0	0
KCl.....		5	21	21	44	60	64
RbCl.....		19	23	26	40	54	43
CsCl.....		3	4	14	9	17	30
NH_4Cl.....		1	0	3	4	17	16

TABLE II.

	Percentage of eggs of *Fundulus* which can develop in M/100 sodium citrate solution if this solution is made up in					
	0	M/40	M/20	M/10	M/5	3 M/10
LiCl.....	0	3	2	0	0	0
NaCl.....	0	6	0	2	1	0
KCl.....	0	26	19	57	52	53
RbCl.....	8	46	55	60	55	42
CsCl.....	8	40	60	56	32	
NH_4Cl.....	8	2	1	2	45	36

The table shows that when the M/100 Na_3 citrate solution is made up in M/5 KCl, RbCl, CsCl, or NH_4Cl practically half the eggs form embryos. In this experiment the addition of the Cl ion may diminish the toxicity of the citrate solution but if this be true the fact remains that the Cl ion can have this effect only when it is added with K or NH_4 ions and not when added with Na or Li ions.

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The NH_4 ion, therefore, resembles in its physiological behavior the K ion more than it does the Na or Li ion.

III. The Antagonism between Li and K.

In any series of ions based on their chemical or physical behavior Na occupies a position between Li and K. Li has a smaller and K has a larger ionic radius than Na. If we replace some of the Na ions of sea water by Li ions we alter the properties of the solution in one sense, and if we replace part of the Na ions by K ions we alter the properties in the opposite sense. We should, therefore, expect that if we replace a certain percentage of Na ions in sea water by Li ions the toxic character of the solution should be diminished if we replace at the same time also a certain percentage of the Na ions by K ions; since with the combined increase of the K ions and of Li ions the effect of Na ions might be more nearly approximated.

The newly fertilized egg of the sea urchin (*Arbacia*) can develop into gastrulæ in an "artificial sea water" of the following composition.

100.0 cc. of M/2 NaCl
1.75 cc. of M/2 $CaCl_2$
2.2 cc. of M/2 KCl
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$

To this was added 0.8 cc. of M/10 $NaHCO_3$ to bring the artificial sea water to a pH of about 7.4.

We prepared the following solution in which M/2 NaCl of the artificial sea water was replaced by M/2 LiCl and which was free from K. Its composition was:

100.0 cc. of M/2 LiCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $NaHCO_3$

This solution, which we will call the Li mixture, permitted us to replace the Na in natural or artificial sea water by Li without altering the constitution of the sea water in any other direction except in regard to K, the concentration of which it was our intention to vary in the experiments.

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Our first experiments consisted in mixing various quantities of natural sea water and Li mixture to find out the maximal amount of Li in natural sea water which still permitted the formation of normal blastulæ in about 16 or 20 hours; the eggs were put into the solution immediately after fertilization. It was found that only 8 per cent of the Li mixture could replace the natural sea water without preventing the development of the eggs into swimming blastulæ. When, however, the proportion of KCl contained in the sea water was increased thirteen times its normal amount the eggs were able to develop into larvæ when as much as 52 per cent of Na was replaced by Li. It is therefore possible to increase the tolerance of the sea urchin egg

TABLE III.

Maximal Amount of Li in which Swimming Blastulæ of Arbacia Can Be Obtained.

K mixture.	Natural sea water.	Li mixture.
cc.	*cc.*	*cc.*
0.0	23.0	2.0
0.5	19.5	5.0
1.0	18.0	6.0
2.0	16.0	7.0
4.0	12.0	9.0
6.0	6.0	13.0

against Li 600 per cent by increasing simultaneously the amount of K normally present in the sea water by 1,300 per cent. Since it was necessary to keep all the other constituents of the sea water constant the KCl was not added in the form of a pure M/2 solution of this salt but in the form of a mixture of the following composition which we will call the KCl mixture.

100.0 cc. of M/2 KCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $KHCO_3$

Systematic experiments showed that the maximum dose of Li in which the eggs could develop into larvæ increased with the concentration of K added to the sea water. This is indicated in Table III.

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The table shows that if we wish to replace in normal sea water more Na by Li we must at the same time also replace an increasing proportion of Na by K.

In order to obtain a more regular curve than expressed in Table III we replaced the natural sea water by a NaCl mixture free from KCl and made up as follows:

100.0 cc. of M/2 NaCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $NaHCO_3$

Experiments were then made to ascertain the maximal amount of Li mixture which permitted the formation of swimming blastulæ for each given amount of KCl. The results are contained in Table IV.

TABLE IV.

Maximal Amount of Li Mixture Permitting Formation of Swimming Blastulæ.

K mixture.	Na mixture.	Li mixture.
cc.	*cc.*	*cc.*
0.1	24.0	1.0
0.2	23.0	2.0
0.5	22.0	3.0
1.0	20.0	4.0
2.0	17.0	6.0
4.0	13.0	8.0
6.0	10.0	9.0
7.0	8.0	10.0

When 8 cc. of K mixture were used no more larvæ were obtained on account of the fact that this concentration of K itself was too toxic.

Table IV shows more clearly than Table III that by replacing more Na ions by K ions we increase at the same time the proportion of Na ions which can be replaced by Li ions, without preventing the development of the sea urchin egg into a swimming larva.

It was then shown that Rb has a similar but not quite so great an effect as K (Table V). RbCl was also added in the form of a mixture containing all the other constituents of sea water except K and Na.

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Cs acts also somewhat like K but still less weakly than Rb. Only one series of experiments was made in an attempt to replace K by Cs, and this series proved that in 2 cc. of M/2 CsCl mixture + 19 cc. of natural sea water + 4 cc. of M/2 Li mixture swimming larvæ could be obtained. When no sea water was replaced by CsCl, 2 cc. of the Li mixture in 25 cc. was the maximum which still permitted the development of larvæ.

These results show that the toxic effects of Li, which occupies in the periodic table a position on one side of NaCl, are mitigated by the addition of ions like K, Rb, and Cs, occupying a position on the other side of Na. A mixture of Li and K ions in proper proportions acts more like a solution of Na ions than do the Li ions alone.

TABLE V.

Maximal Amount of Li Mixture Permitting Formation of Swimming Larvæ.

Rb mixture.	Sea water.	Li mixture.
cc.	*cc.*	*cc.*
1.0	20.0	4.0
2.0	18.0	5.0
4.0	14.0	7.0

It should be taken into consideration that in these experiments the balance between monovalent and bivalent cations was not disturbed.

If we replace a smaller or larger percentage of the Na ions contained in sea water by Mg or by Ca ions the toxicity of LiCl is not diminished. This is probably due to the fact that the quantity of Ca or Mg required for balancing the monovalent cations is naturally present in the sea water.

The antagonism between LiCl and KCl can also be demonstrated in the eggs of *Fundulus*. When newly fertilized eggs of *Fundulus* are put into M/5 LiCl all the eggs are dead before an embryo is formed. If, however, the M/5 solution of LiCl also contains a small quantity of RbCl or of KCl, as many as 20 per cent of the eggs live long enough to form embryos. When, however, the M/5 LiCl solution contains NaCl not a single embryo is formed. CsCl also gives a positive effect. The M/5 LiCl solution was made up in distilled water and in different

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concentrations of the salts mentioned. Table VI gives the results of an experiment. The first horizontal row gives the molecular concentration of NaCl, KCl, RbCl, and CsCl in which the M/5 solution of LiCl was made up. The figures in the next horizontal rows give the percentage of eggs which formed embryos.

The table shows that the addition of NaCl did not protect the *Fundulus* eggs against the toxic effects of LiCl, while the KCl, RbCl, and CsCl had a protective or antagonistic effect. The protective effect of these salts is considerably less than that produced by a salt with a bivalent cation since in the latter case 80 per cent or more of the eggs form embryos in a M/5 solution of LiCl.

TABLE VI.

	Percentage of eggs which formed embryos in M/5 LiCl made up in					
	0	M/40	M/20	M/10	M/5	3 M/10
NaCl.....	0	0	0	0	0	0
KCl.....	0	18	10	14	11	6
RbCl.....	0	7	13	18	20	2
CsCl.....	0	2	0	5	9	2

These examples may suffice to show that the action of the potassium ion in the phenomena of antagonism (which underlie the physiological balance of ions in salt solutions) is in agreement with the purely chemical character of this ion, *i.e.* its position in the periodic table; and that hence there is no reason to attribute its physiological action in these cases to some other factor; *e.g.*, its extremely minute radioactivity.

SUMMARY.

1. It is shown that the NH_4 ion acts in cases of antagonism on the egg of *Fundulus* more like the K ion than the Na ion; this corresponds to the fact that in its general chemical behavior the NH_4 ion resembles the K ion more closely than the Na ion.

2. It is shown that the tolerance of sea urchin eggs towards the Li ion can be increased 500 per cent or more if at the same time a certain amount of Na ion is replaced by K, Rb, or Cs ions. Since in the

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periodic table Na occupies a position between K and Li it is inferred that the Li and K ions deviate in their physiological action in the opposite direction from the Na ion.

3. These data indicate that the behavior of the K ion in antagonistic salt action (which forms the basis of the physiologically balanced action of ions) is due to its purely chemical character, *i.e.* its position in the periodic table or rather to its atomic number, and not to those explosions in its nucleus which give rise to a trace of radioactivity.

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ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. II.

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I. Combining Ratios of Acids and Bases with Gelatin and the Swelling of Gelatin.

In this paper we will continue the demonstration of the relation between the combining ratios of acids and bases with proteins and the effect of ions on the physical properties of proteins. This demonstration completes the proof that the purely chemical forces of primary valency determine the reactions of proteins with other compounds.

It is generally stated in colloidal literature that gelatin swells more in chlorides, bromides, or nitrates than in water and that it swells less in citrates, acetates, tartrates, phosphates, and sulfates than in water. The author of this statement is Hofmeister[1] who was a pioneer in this work and who cannot be blamed for not having considered certain sources of error in his methods. In Hofmeister's experiments gelatin blocks were put into salt solutions of so high a concentration that—as we now know—no specific ion effects could be expected and the slight differences in swelling actually observed by him were probably merely accidental. He even mentions that sugar solutions have a "dehydrating" effect, and this fact alone should have warned chemists against using his experiments for conclusions concerning the specific effects of ions on the physical properties of colloids. As far as the writer can determine from the literature the discrimination between "hydrating" and "dehydrating" ions originated from these experiments.

[1] Hofmeister, F., *Arch. exp. Path. u. Pharmakol.*, 1891, xxviii, 210.

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It is often asserted that Hofmeister's ion series for swelling has been confirmed by other authors. Thus Zsigmondy[2] makes the following statements in support of this impression.

"Wo. Ostwald who compared the efficiency of different acids found that swelling diminishes in the acids in the following order,

$$HCl > HNO_3 > \text{acetic acid} > \text{sulfuric acid} > \text{boric acid.}$$

Fischer has shown that the acid and alkali swelling of gelatin as well as that of fibrin is diminished by the addition of salt, and that chlorides, bromides, and nitrates have a less dehydrating action than acetates, sulfates, or citrates. We have here a similar series as in the case of the precipitation of proteins by alkali salts, although the order does not agree entirely."

The writer is inclined to interpret Ostwald's and Fischer's experiments differently from Zsigmondy, since both authors ignored the hydrogen ion concentration of their solutions. We believe to have shown that it is necessary to base conclusions concerning the relative efficiency of ions on experiments with equal hydrogen ion concentration. By ignoring this postulate Ostwald has only succeeded in proving that acetic and boric are weaker acids than nitric but not that gelatin swells less in acetates or borates than in nitrates; and Fischer has only succeeded in proving that citrates and acetates are buffer salts which when added to a solution of a strong acid diminish its hydrogen ion concentration, but not that acetates and citrates diminish the swelling of gelatin. These authors attributed the effects caused by a variation in the hydrogen ion concentration of their solutions erroneously to an influence of the anion. The Hofmeister series of ion effects on swelling has,in reality, never been confirmed.

If we wish to study the specific effects of ions on the swelling of gelatin we must proceed from isoelectric gelatin, bring it to different pH values by different acids or alkalies, and then compare the swelling at the same pH for these different acids or alkalies. If this is done it is found that when gelatin is in combination with the anion of a weak dibasic or tribasic acid, *e.g.* tartaric, citric, phosphoric, its degree of swelling is practically the same as when it is in combination with Cl or NO_3; since in all these cases the anion of the gelatin salts is monovalent. Only in the case of gelatin sulfate is the swelling considerably less,

[2] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd edition. 1918, 373

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because the anion is divalent, H_2SO_4 combining with gelatin in equivalent and not in molecular proportions as do the weak dibasic or tribasic acids; *e.g.*, tartaric or phosphoric.

A few words are necessary concerning the method of these experiments. We can measure the amount of swelling by determining the increase in weight of a given mass of gelatin or by determining its increase in volume. We have adopted the following simple and quick volumetric method (although we intend to supplement these experiments later with gravimetric experiments).

Dry powdered gelatin, of pH = 7.0, was sifted and the grains no longer going through Sieve 50 but going through Sieve 40 or 30 were selected for the experiment. We had therefore fairly uniform grains of not too small a diameter. Doses of 1 gm. each of such powder were weighed out, each dose was put for an hour into 100 cc. of M/128 acetic acid at 10°C. to bring the gelatin to the isoelectric point. The powdered mass was then put on a filter and washed five times with 25 cc. of distilled water at 5°C. In the acetic acid solution and during the washing on the filter the powdered gelatin is stirred constantly.

Each dose of originally 1 gm. of dry powder which had meanwhile absorbed a certain quantity of liquid (which was about the same for each gram of the isoelectric powder) was then put for 1 hour at about 20° into 100 cc. of different concentrations of the acid or base whose influence on swelling was to be tested, and the suspension was constantly agitated. It was found that in an hour the granules of gelatin had reached the maximal swelling in each solution. To measure the relative amount of swelling in different acids or alkalies and at different pH the suspension was poured into graduate cylinders of 100 cc. each (and all of the same diameter) in which the granules fell very rapidly to the bottom. The cylinders were kept in a water bath at 20°C. for about 10 to 15 minutes and the volume occupied by the gelatin granules was then read. This volume included a certain amount of solution between the granules and therefore the real volume of the gelatin was smaller than that read. While therefore the method cannot be used to measure the absolute amount of swelling it allowed us to determine the relative influence of different acids or bases on the swelling for the same pH.

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The determination of the pH of the gelatin in these experiments requires a short discussion since the pH inside the gelatin is quite different from that in the supernatant liquid, owing to the Donnan equilibrium. Donnan has shown that when a solution of a colloidal salt is separated from the solution of a crystalloidal salt with a common ion by a membrane which is permeable for the crystalloidal but not for the colloidal ions the concentration of the crystalloidal salt is, at the point of equilibrium, always lower on the side of the colloidal solution than on the side of the crystalloidal solution.[3] This was invariably the case in our experiments on osmotic pressure reported in the preceding paper. When, for example, a gelatin chloride solution of pH 3.5 was put inside a collodion bag and the latter was dipped into a solution of HCl (without gelatin) also of a pH 3.5, the pH on the two sides of the membrane did not remain the same since some of the free acid was forced from the colloidal solution into the pure acid solution outside the collodion bag, so that the pH of the outside solution fell while that of the inside rose.

As Procter[4] has pointed out this Donnan equilibrium must play a rôle also in the case of the swelling of gelatin since in this case the surface of the gelatin granule takes the place of the membrane permeable for the crystalloidal electrolyte but not for the colloid.

In our experiments 1 gm. of originally isoelectric gelatin was put for 1 hour at 20°C. into 100 cc. of acid, *e.g.* HCl, of different concentration varying from M/16 to M/8,192. After an hour equilibrium was reached and the pH of the supernatant fluid was determined. The gelatin was put on a filter (after the volume of the gelatin in the graduate cylinder had been measured) and all the acid was allowed to drain off. A trace of outside acid probably remained on the surface of each granule though presumably some of the free acid inside the granule diffused to the surface under the influence of pressure. This error was partly but not completely compensated by adding enough distilled water of pH of about 5.6 to the gelatin after it had

[3] Donnan, F. G., *Z. Electrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[4] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

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been melted to bring the volume to 100 cc. The pH of the 1 per cent solution of originally isoelectric gelatin was determined colorimetrically. It was found that the pH of the supernatant HCl solution was always considerably smaller than the pH of the gelatin solution (Table I).

The first row in Table I gives the molecular concentration of the 100 cc. of HCl into which the gelatin was originally put. The second row gives the pH of these supernatant HCl solutions after 1 hour, and the third row gives the pH of the gelatin solutions after the supernatant HCl solution had been drained off and after the remaining mass of gelatin had been melted and brought to a volume of 100 cc. by adding enough distilled water of pH 5.6. It will be noticed: first, that the pH of the supernatant HCl solution after 1 hour is higher than the pH of the original HCl solution owing to the fact that some acid combined with the gelatin; and, second, that the pH of the gelatin solution is considerably higher than that of the supernatant solution owing to the Donnan equilibrium, according to which the concentration of free acid outside the gelatin must be greater than inside.

In order to get the correct difference due to the Donnan equilibrium, solutions of gelatin salts were put into collodion bags and these bags were dipped into beakers containing 350 cc. of solution of the same acid or base as that inside the collodion bag and possessing the same pH as the gelatin solution; the outside solution, of course, was free from gelatin. In the case of gelatin-acid salts free acid invariably diffused from the gelatin solution into the pure acid solution, *e.g.* HCl, so that the pH of the latter became smaller and that in the gelatin solution higher. In these experiments the volume of the outside pure HCl solution was 350 cc. and that of the inside 1 per cent gelatin solution only 50 cc. Table II gives the result of one experiment of this kind.

Thus at equilibrium pH the gelatin solution was 3.8 and the outside solution 3.2, or inside 3.3, and outside 4.0. This difference is in the same sense and of nearly but not quite the same order of magnitude as that observed in Table I.

We may therefore conclude that the real pH of the gelatin solution inside the granules of gelatin was slightly less than that measured by our method.

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TABLE I.

Original concentration of supernatant HCl solution.	M/16	M/32	M/64	3M/256	M/128	3M/512	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192
pH of the supernatant HCl solution after 1 hr..	1.25	1.6	1.9	2.1	2.35	2.55	2.8	3.2	3.4	3.6	3.85	4.0
pH of 1 per cent gelatin solution after 1 hr.....	2.2	2.3	2.7	3.0	3.25	3.4	3.8	4.2	4.6	4.65	4.7	4.7

TABLE II.

pH at beginning of experiment, inside and outside.....	2.3	2.6	3.0	3.1	3.3	3.5	3.6	3.75	4.1	4.38	4.8
pH of outside HCl solution after 20 hrs.....	2.1	2.5	2.9	3.0	3.15	3.2	3.3	3.4	3.7	4.0	4.8
pH of gelatin solution after 20 hrs.....	2.6	3.2	3.3	3.5	3.7	3.8	4.0	4.1	4.2	4.4	4.8

TABLE III.

Original concentration of supernatant KOH solution.....	M/32	M/64	M/128	M/256	M/512	7M/4,096	6M/4,096	5M/4,096	M/1,024	M/2,048	M/4,096	M/8,192
pH of the supernatant KOH solution after 1 hr..	12.4	12.0	11.6	11.3	8.0	7.2	7.0	6.6	6.6	5.8	5.1	5.2
pH of 1 per cent gelatin solution after 1 hr.....	12.0	11.6	11.0	9.8	6.3	5.7	5.5	5.4	5.3	5.1	4.9	4.8

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The results of our experiments on swelling are expressed in Figs. 1, 2, and 3. The abscissæ in Fig. 1 are the pH found in the gelatin after equilibrium was established. The ordinates represent the figures for the volume of the granules of 1 gm. of gelatin in different acids. It is obvious that in all cases the volume (or swelling) is a

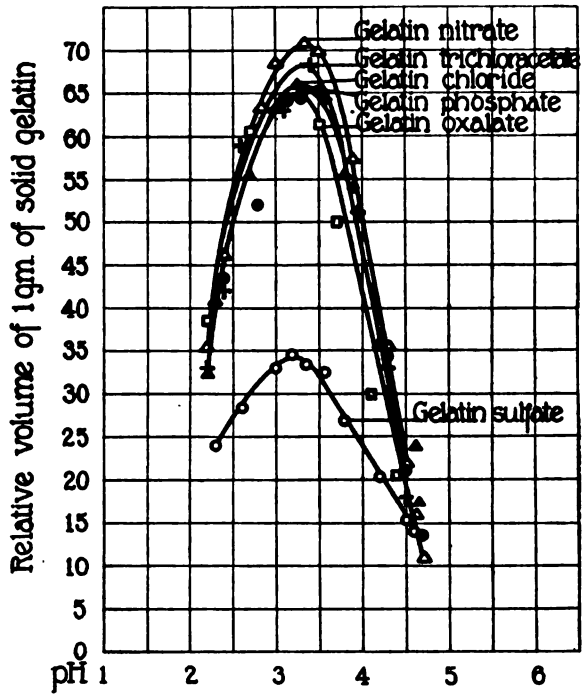

FIG. 1. Influence of HCl, HNO_3, H_3PO_4, H_2SO_4, trichloracetic, and oxalic acids on the swelling of gelatin. Abscissæ are the pH, ordinates the volume of gelatin. The curves for all the acids are practically identical except that for H_2SO_4 which is about one-half as high as the curves for the other acids.

minimum at the isoelectric point pH = 4.7, that it rises with diminishing pH until the maximum is reached at a pH of about 3.2 or 3.3, and that the curve drops steeply with a further diminution of pH (*i.e.* a further increase of hydrogen ion concentration). The fact that the maximum lies here at pH of about 3.2, while in our osmotic pressure curves it was at about 3.3 or 3.4, indicates the degree of error in

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the measurement of pH in this case due to the adhesion of some of the original acid on the outside of the granules. This error was partly compensated by the addition of distilled water of pH of about 5.6 in making up the 1 per cent solution of gelatin. On the whole the

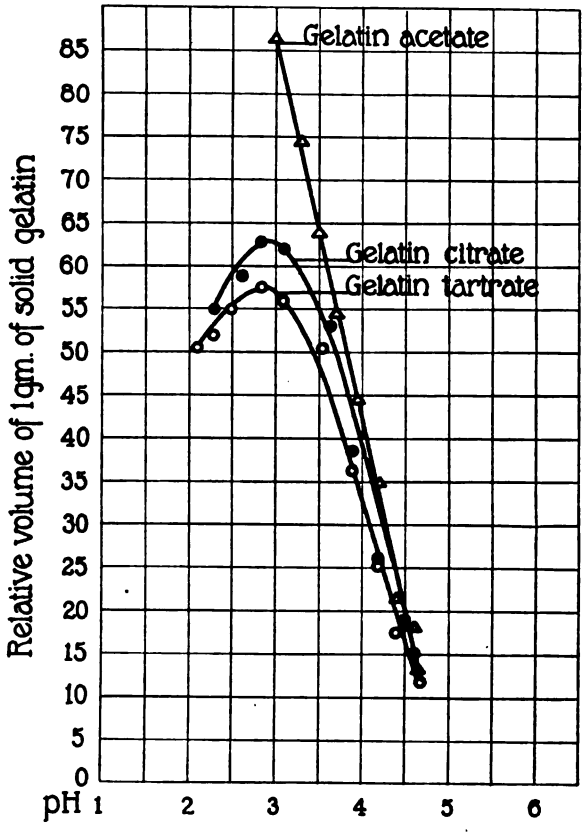

FIG. 2. Influence of citric, tartaric, and acetic acids on swelling of gelatin. The curves for citric and tartaric acids are practically identical with those for HCl and HNO_3 in Fig. 1. That for acetic acid is a little higher owing probably to some specific and secondary effect of this acid.

probable error was +0.1 or +0.2; *i.e.*, the real pH was 0.1 or at the utmost 0.2 greater than in our abscissæ. The most important fact is, however, that the curves for the influence of HCl, HNO_3, trichloracetic, oxalic, phosphoric, citric, and tartaric acids are practically

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identical (Figs. 1 and 2), proving that only the effect of the valency and not that of the nature of the anion of the acid used influences the swelling; since we have seen that the anion of weak dibasic or tribasic organic acids combining with the gelatin is always monovalent.

The curve for the swelling of gelatin sulfate, where the anion combining with gelatin is bivalent, is only half as high as the curve for the salts of gelatin with the anion of weak dibasic acids (Figs. 1 and 2).

Acetic acid gives an increasing amount of swelling (Fig. 2), but it must be remembered that M/1 acetic acid had to be used to bring the pH of the gelatin to 3.0, and it is not impossible that in this case a secondary chemical or physical modification of the gelatin may complicate the conditions.

It is of interest to compare these curves with those which should be expected according to the Hofmeister series. In the latter case the curves for phosphate, oxalate, citrate, tartrate, and acetate should coincide with the curve for sulfate instead of coinciding with the curves for Cl and NO_3. This difference is due to the fact that the believers in the Hofmeister series did not determine the pH and that they erroneously ascribed the effects due to a variation in the hydrogen ion concentration to a difference in the influence of the anion.

The ratio between the effects of sulfuric acid on swelling and that of the other acids is again not far from 1:2. If we deduct the swelling of the isoelectric gelatin (of about 10 mm.) from the values of our ordinates the swelling of gelatin sulfate at pH of about 3.3 is less than one-half that of the other gelatin-acid salts where the anion in combination with gelatin is monovalent.

When powdered isoelectric gelatin is treated with an alkali, *e.g.* KOH, the supernatant watery solution is less acid or more alkaline than the gelatin granules. The CO_2 of the air lowers the pH of the solutions a little but this error affects the pH of the supernatant watery solution more than it does the gelatin which has a buffer action. Table III, p. 252, gives the original concentration of the watery solution of KOH, into 100 cc. of which 1 gm. of powdered isoelectric gelatin was put (first row, Table III). After 1 hour the pH of the supernatant watery solution was determined (second row, Table III);

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the supernatant solution was drained off from the gelatin, the latter melted, and the volume brought to 100 cc. by adding distilled water of pH of about 5.6, and the pH of the gelatin solution was determined (third row, Table III).

It is obvious that the pH of the supernatant solution is higher than that of the gelatin solution, as we should expect from the Donnan equilibrium.

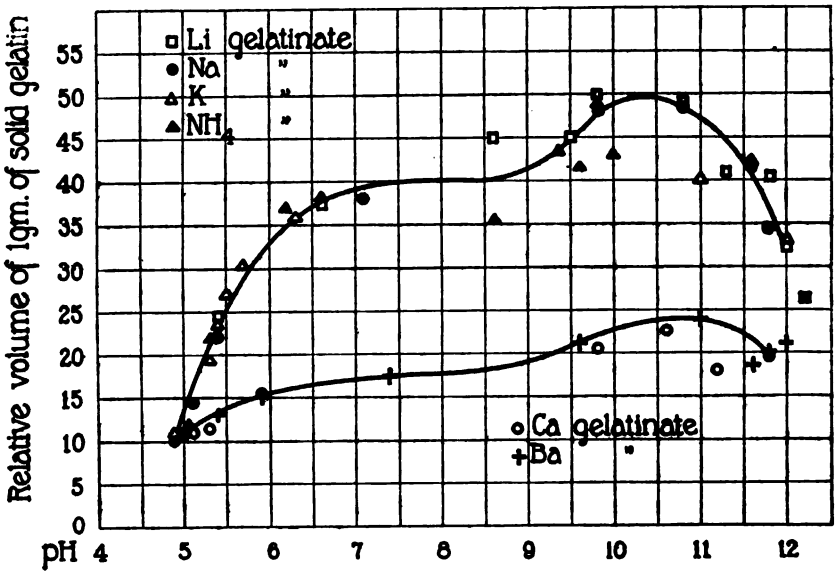

FIG. 3. Curves for the effect of different bases on swelling. Those for LiOH, NaOH, KOH, and NH_4OH are practically identical and about twice as high as those for $Ca(OH)_2$ and $Ba(OH)_2$.

Fig. 3 gives the curves for the action of alkalies on swelling. The curves for Li, Na, K, and NH_4 gelatinate of the same pH are practically identical, except that the values for NH_4OH are irregular for pH above 8.5 possibly on account of the fact that the concentration of NH_4OH required to bring gelatin to such pH is rather high. The main fact is that the ratio of the maximal swelling of gelatin salts with bivalent cation like Ca or Ba is half or possibly a little less than half of that of gelatin salts with monovalent cation, like Na, K, or NH_4.

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Near pH = 7.0 the curves run parallel to the axis of abscissæ for the reason that a considerable variation in pH signifies only a negligible change in the concentration of gelatin salt formed. The experiments were not carried beyond a pH of 12.0 on account of the lack of reliable indicators for that region, and on account of the fact that alkali causes chemical changes in the gelatin.

It should be pointed out that the maximal swelling of gelatin in alkalies was less than that in acids. This was not observed in the osmotic pressure curves.

II. Relative Solubility of Different Gelatin Salts in Mixtures of Water and Alcohol.

When powdered gelatin is brought to the isoelectric point, melted, and made into a 1 per cent solution it is at first transparent. After some time, which is the shorter the lower the temperature, the gelatin solution becomes opaque; and in the course of weeks or months it may settle in the form of a precipitate. This, however, does not happen in each case, possibly for the reason that the precipitation will occur only at a very definite pH, while, with a slight deviation from this point in either direction, the result will be only an opacity at room temperature. Raising of the temperature will again result in the clearing of the opacity. The opacity seems therefore to be due to the formation of larger aggregates of protein molecules and these will float as long as they are not too large. The setting of the solution to a gel is a different process from this precipitation since no cloudiness or opacity needs to be connected with this latter phenomenon.

When we add to a freshly prepared solution of isoelectric gelatin only a trace of 95 per cent alcohol the cloudiness which would have formed slowly is noticed at once and if we add a little more alcohol we can produce at once a dense precipitate. In order to standardize the degree of cloudiness produced we add so much 95 per cent alcohol to 10 cc. of a 1 per cent solution of isoelectric gelatin in a test-tube of definite diameter until certain letters become illegible when looked at through the test-tube filled with the gelatin-alcohol-water mixture. Since the addition of alcohol to the watery solution raises the temperature and since this has the tendency to diminish the degree of

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opacity of the mixture it was necessary to dip the test-tube in ice water during the process of mixing and keep the gelatin solution approximately at 10°C.

When we prepare gelatin chloride by adding small quantities of HCl to isoelectric gelatin we need the more alcohol the lower the pH and very soon a limit is reached when the addition of 25 cc. or more alcohol no longer brings about any precipitate or even cloudiness. Thus 10 cc. of isoelectric gelatin required 2 cc. of 95 per cent alcohol to bring about that high degree of opacity at which the test letters were no longer legible. When the pH of the gelatin was lowered to 4.55

TABLE IV.

Cc. of 95 per cent alcohol required to bring 10 cc. of 1 per cent gelatin-salt solution to standard opacity.

	pH of gelatin-acid salt.									
	4.6	4.55	4.45	4.4	4.2	3.75	3.3	3.1	2.6	2.1
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Gelatin chloride		5.5	∞	∞	∞	∞	∞	∞	∞	∞
" oxalate	5.3	18.1		∞	∞	∞	∞	∞	∞	∞
" phosphate		28.0		∞	∞	∞	∞	∞	∞	∞
" tartrate		11.6		∞	∞	∞	∞	∞	∞	∞
" succinate		3.0	5.4	∞	∞	∞	∞	∞	∞	∞
" citrate		4.2		∞	∞	∞	∞	∞	∞	∞
" acetate		2.7	20.0	∞	∞	∞	∞	∞	∞	∞
" monochloracetate		4.1	∞	∞	∞	∞	∞	∞	∞	∞
" dichloracetate		5.9	∞	∞	∞	∞	∞	∞	∞	∞
" trichloracetate	2.9			∞	∞	∞	∞	∞	∞	∞
" sulfate		6.8		9.0	11.2	12.8	14.6	14.4	13.2	13.3

by the addition of HCl, 5.5 cc. of alcohol were required for the same degree of opacity. When the pH of the 1 per cent gelatin chloride solution was only a trifle lower, namely 4.50, the addition of 25 cc. of alcohol or more did not suffice for bringing about the degree of opacity required for our test; only a lower degree of turbidity resulted. A gelatin chloride solution of pH 4.45 remained perfectly clear (with a bluish tint) regardless of how much alcohol was added. We may say that gelatin chloride becomes soluble in an alcohol-water mixture containing more than 75 per cent alcohol as soon as its pH is $\leqq$ 4.45.

It seemed of interest to compare the relative solubility of other gelatin-acid salts with that of gelatin chloride. Table IV gives the

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result. The figures indicate the number of cc. of 95 per cent alcohol which when added to 10 cc. of 1 per cent gelatin solution brings about the standard degree of opacity. When the addition of 30 cc. or more alcohol to 10 cc. of the 1 per cent solution of gelatin-acid salt leaves the solution perfectly clear we indicate this by the sign ∞.

The result (which agrees with the results of a previous publication by the writer[5]) is unequivocal: all those gelatin-acid salts in which the anion in combination with gelatin is monovalent can no longer be precipitated by 95 per cent alcohol when the pH is ≷ 4.4; while the only gelatin-acid salt in combination with a bivalent anion, namely gelatin sulfate, can be precipitated at any pH down to 2.0 (or even below). The relative solubility of gelatin-acid salts in alcohol shows,

TABLE V.

Cc. of 95 per cent alcohol required to bring 10 cc. of 1 per cent gelatin-salt solution to standard opacity.

	pH of metal gelatinate.							
	4.9	5.0	5.4	6.4	9.6	10.2	11.4	12.0
	cc.	*cc.*	*cc.*	*cc*	*cc.*	*cc.*	*cc.*	*cc.*
Li gelatinate.....	2.3	∞	∞	∞	∞	∞	∞	∞
Na "	2.0		∞	∞	∞	∞	∞	∞
K "	1.9	∞	∞	∞	∞	∞	∞	∞
NH_4 "	1.9	∞	∞	∞	∞	∞	∞	∞
Ca "		2.8	4.4	8.2	8.2	10.5	12.0	7.2
Ba "		2.1	4.2	6.6	7.9	8.1	5.9	5.3

therefore, the same influence of the valency (and lack of influence of the nature of the anion) which we have found in connection with the other properties of proteins like swelling, osmotic pressure, and viscosity.

The same agreement exists in regard to metal gelatinates. 10 cc. of a 1 per cent solution of Li, Na, K, and NH_4 gelatinate can no longer be precipitated by the addition of 95 per cent alcohol when the pH is ≶ 5.0. The deviation from the isoelectric point is minute. 10 cc. of 1 per cent Ba and Ca gelatinate, however, can be precipitated with comparatively small quantities of 95 per cent alcohol at any pH (Table V).

[5] Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 489.

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We should expect that when the hydrogen ion concentration of a gelatin chloride solution becomes very high its solubility in an alcohol-water mixture will be diminished again. This is indeed the case, and happens when in 100 cc. of 2 per cent solution of isoelectric gelatin are contained 30 or 40 cc. of M/1 HCl. When to 5 cc. of such a solution are added 25 or 20 cc. of 95 per cent alcohol, the turbidity occurs again. When 100 cc. of the solution contain 50 cc. of M/1 HCl only 14.7 cc. of 95 per cent alcohol are required.

The same result was obtained with Na gelatinate which can also be precipitated again by alcohol when its pH exceeds 12 or 13.

The fact that the gelatin-acid salts (with the exception of gelatin sulfate) become completely soluble in alcohol when the pH reaches the low value of 4.4 is not easy to harmonize with the hypothesis of Pauli that this is due to the ionization of the gelatin, since the relative amount of ionized gelatin is exceedingly small at pH 4.4.

The experiments on the relative solubility of different gelatin salts therefore show the same influence of the valency of the ion in combination with gelatin as was shown in regard to the other physical properties of proteins.

III. Conductivity and Ionization of Gelatin Solutions.

The influence of ions on the conductivity of protein solutions should run parallel to the influence on swelling, viscosity, and osmotic pressure, if it be true that these properties depend on the concentration of the protein ions in the solution. According to this theory, first proposed by Laqueur and Sackur[6] and elaborated by Pauli,[7] the values for the physical properties of proteins are a minimum at the isoelectric point for the reason that the ionization of the protein molecules is a minimum at that point. When we add acid, *e.g.* HCl, protein chloride is formed which is highly ionized and the increase in the viscosity, swelling, and osmotic pressure with the increase of acid is explained by the ionization theory on the assumption of an increase in the concentration of the protein ions in the solution. When, however, too much acid is added, *i.e.* as soon as the pH of the gelatin solution

[6] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

[7] Pauli, W., Kolloidchemie der Eiweisskörper, pt. 1, Dresden and Leipsic, 1920.

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falls below 3.3, the swelling, osmotic pressure, and viscosity of the solution diminish again upon the addition of further acid. This would be explained by the ionization theory on the assumption that the concentration of ionized protein in the solution reaches a maximum at a pH of about 3.3, and that a further increase of acid lowers the concentration of ionized gelatin in the solution. The same theory should also explain the fact that the curves for the physical properties of gelatin salts with a bivalent ion are so much lower than the gelatin salts with a monovalent ion by the assumption that the latter are more highly ionized than the former.

We can determine the concentration of ionized gelatin in solution with the aid of conductivity measurements of the solution of a gelatin salt, *e.g.* gelatin chloride, if we deduct the conductivity of the free HCl in the solution from the total conductivity of the gelatin solution, since our gelatin solutions contain no other electrolyte except the free acid, *e.g.* HCl, and the gelatin salt; *e.g.* gelatin chloride. This is proved by the fact that at the isoelectric point our gelatin solutions had practically the conductivity zero (Figs. 4, 6, 8, and 9). Our method of procedure was as follows: doses of 1 gm. of powdered gelatin were brought to the isoelectric point and to each gram of isoelectric gelatin were added different quantities of 0.1 N acid or alkali and some water; the mass was melted by heating to 40° and then so much H_2O was added that the volume of the solution was 100 cc. After that the pH of the gelatin solution and the conductivities were determined.

Fig. 4 gives the curves for such measurements in the case of gelatin chloride. The abscissæ are the pH, the ordinates the specific conductivities multiplied by 10^4. The curve to the right is the total specific conductivity $\times 10^4$ of the gelatin chloride solution of different pH. The curve to the left represents the measurements of the specific conductivities $\times 10^4$ of pure HCl solutions (without gelatin) for different pH. By deducting the ordinates of this latter curve from the ordinates of the curve for total conductivity we get the curve in the middle representing the specific conductivity $\times 10^4$ of the pure gelatin chloride solution. Since it had been shown before that the viscosity of the solution does not influence the conductivity in this case (Hardy,

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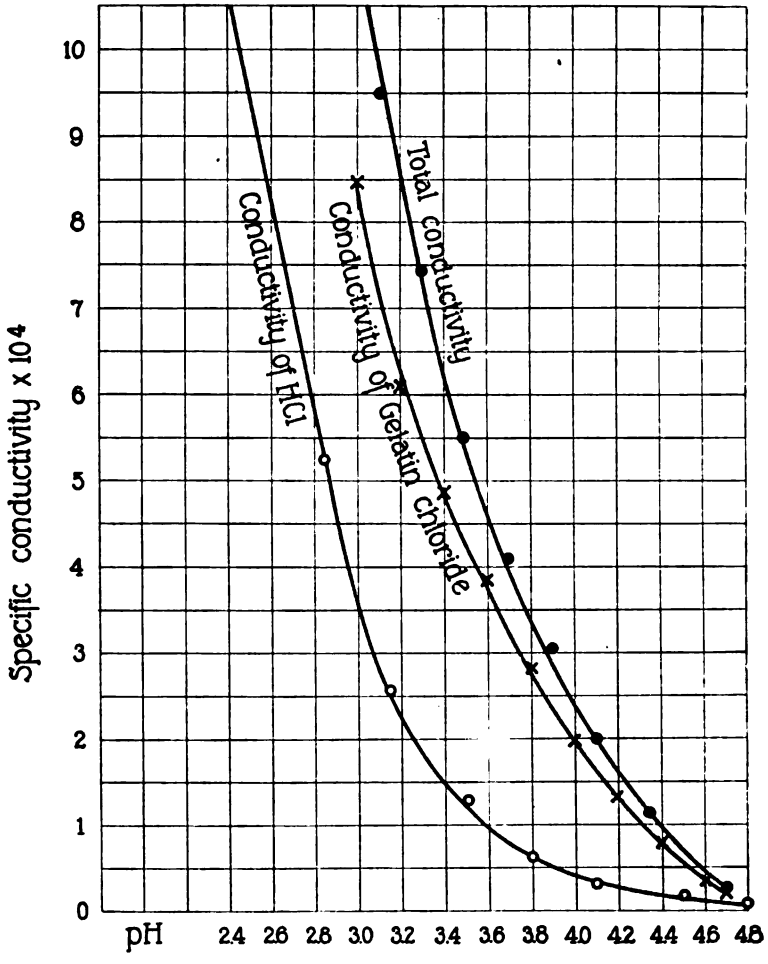

FIG. 4.

FIGS. 4 and 5. Specific conductivity of gelatin chloride solutions of different pH (but all 1 per cent in regard to isoelectric gelatin). Abscissæ are the pH, ordinates specific conductivity $\times 10^4$. Total conductivity means specific conductivity $\times 10^4$ of the gelatin solution measured directly. From this is to be deducted the specific conductivity of HCl of the same pH as the gelatin solution, to obtain the real curve for the specific conductivity of gelatin chloride.

Loeb, Northrop[8]), we may conclude that the middle curve represents the specific conductivity of the gelatin chloride solution and that it

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 605.

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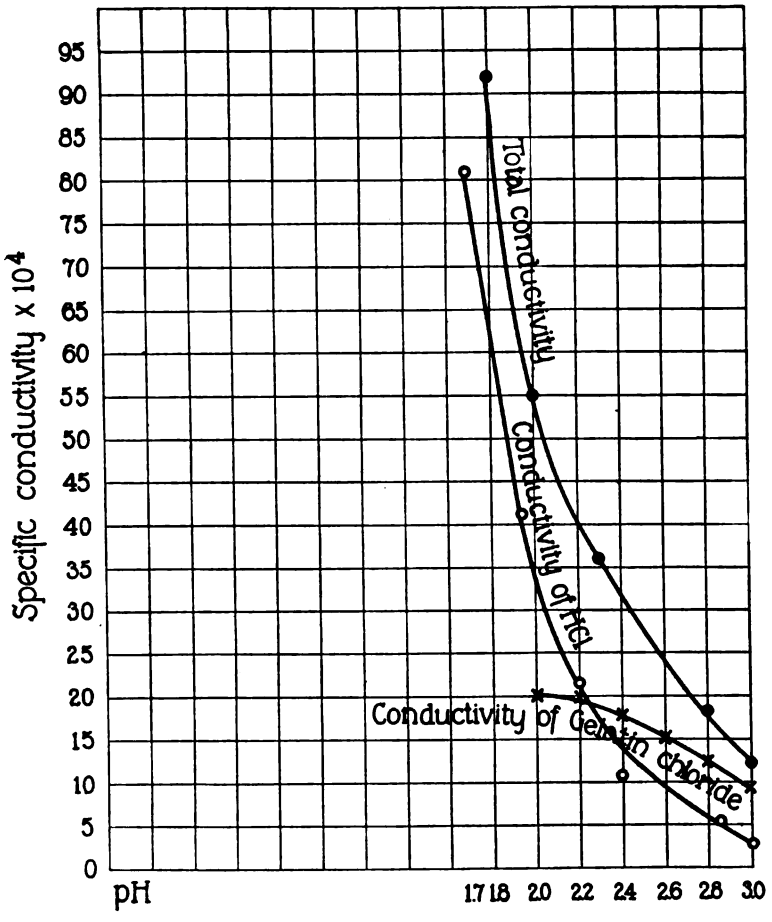

FIG. 5.

can hence be used as a measure of the concentration of the ionized gelatin in the solution. Fig. 4 shows that the curve for the conductivity of gelatin chloride rises continually with increasing hydrogen ion concentration. Fig. 5 is a completion of Fig. 4 for pH down to 2.0. (The ordinates are on a smaller scale in Fig. 5 than in Fig. 4.) It is obvious that at no time does the conductivity curve for gelatin chloride, *i.e.* the curve representing the concentration of ionized protein, show the drop observed in the curves representing the other properties of proteins.

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Figs. 6 and 7 show that the same is true for the conductivity curve for gelatin sulfate; Fig. 6 gives the specific conductivities for pH 4.7 to 3.0, and Fig. 7 for pH 3.0 to 2.2 (the ordinates in Fig. 7 are on a

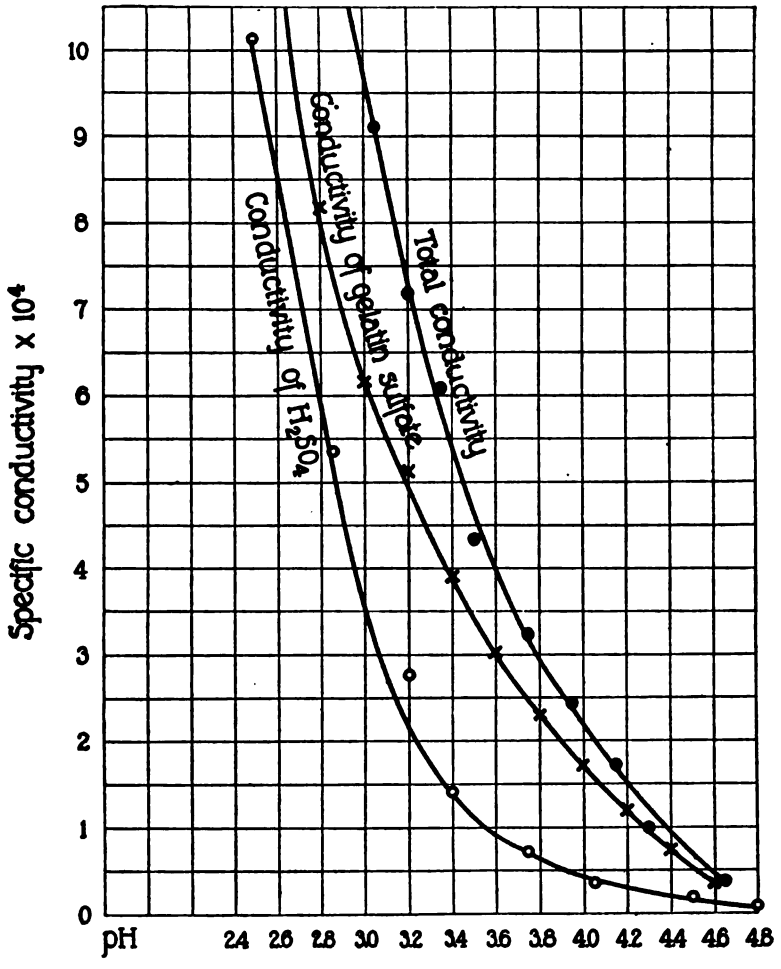

FIG. 6.

FIGS. 6 and 7. Conductivity curves for gelatin sulfate. See legend for Figs. 4 and 5.

smaller scale than in Fig. 6). Experiments on the conductivity of gelatin acetate, trichloracetate, phosphate, and oxalate all give a

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similar result. These experiments do not support the hypothesis that the drop in the curves for viscosity, swelling, and osmotic pressure of gelatin-acid salts at or near pH 3.3 is due to a corresponding drop in the degree of ionization of the gelatin salts mentioned.

No drop was discovered in the conductivity curves for metal gelatinates (Na gelatinate, Fig. 8, and Ba gelatinate, Fig. 9).

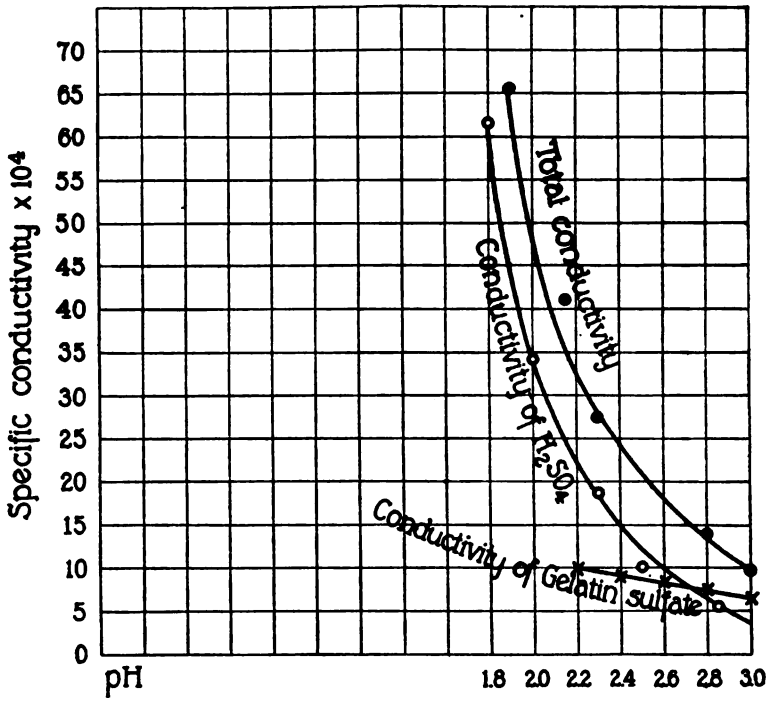

FIG. 7.

The question now arises whether we can explain the difference in the swelling, osmotic pressure, and viscosity of gelatin sulfate on the one hand and gelatin chloride and oxalate, etc., on the other hand on the basis of the ionization theory. If the ionization theory is correct the conductivity of gelatin oxalate, and of gelatin chloride should be twice or almost two and one-half times as great as that of gelatin sulfate. Yet Table VI shows that there is very little difference

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between the conductivities of gelatin oxalate and gelatin sulfate; and also a difference of only 20 per cent between gelatin sulfate and gelatin chloride at pH 3.7. As a matter of fact the difference in conduc-

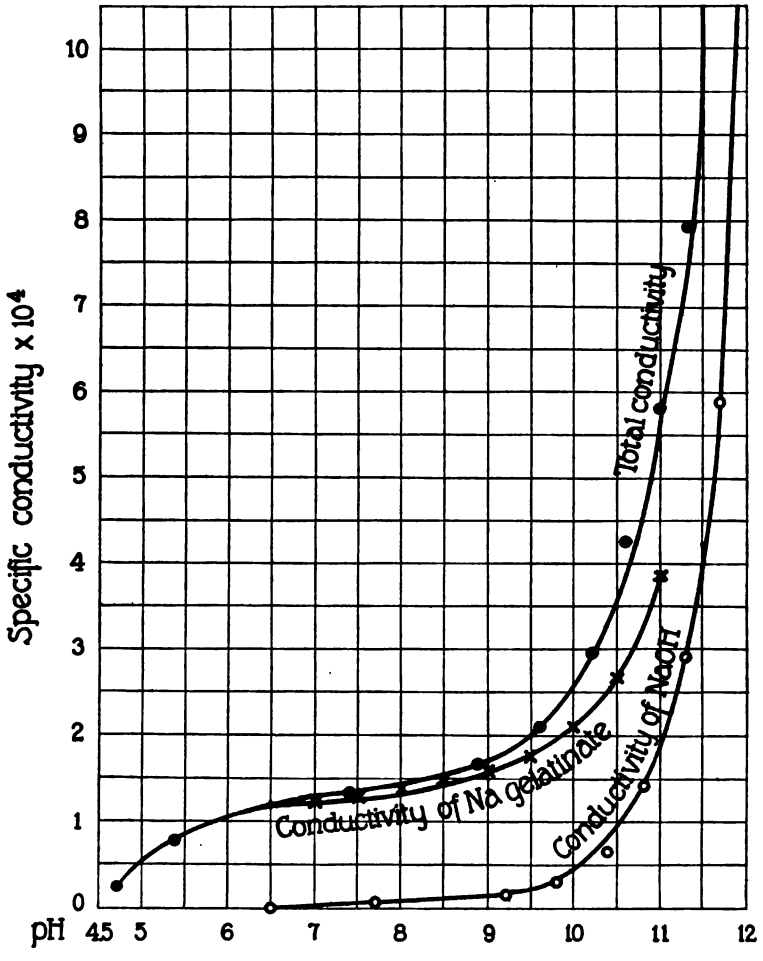

FIG. 8. Conductivity curve for Na gelatinate.

tivity between gelatin oxalate and gelatin chloride which show equal swelling, viscosity, and osmotic pressure is greater than the difference in conductivity between gelatin chloride and gelatin sulfate which are so enormously different in regard to swelling, osmotic pressure, etc.

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The three salts, gelatin chloride, sulfate, and oxalate were chosen, since the ionic mobilities of Cl, $\frac{1}{2}$ SO_4, and $\frac{1}{2}$ oxalate are so nearly alike.

It had been pointed out by the writer in a previous paper that the difference in conductivities of Na and Ba gelatinate and of gelatin

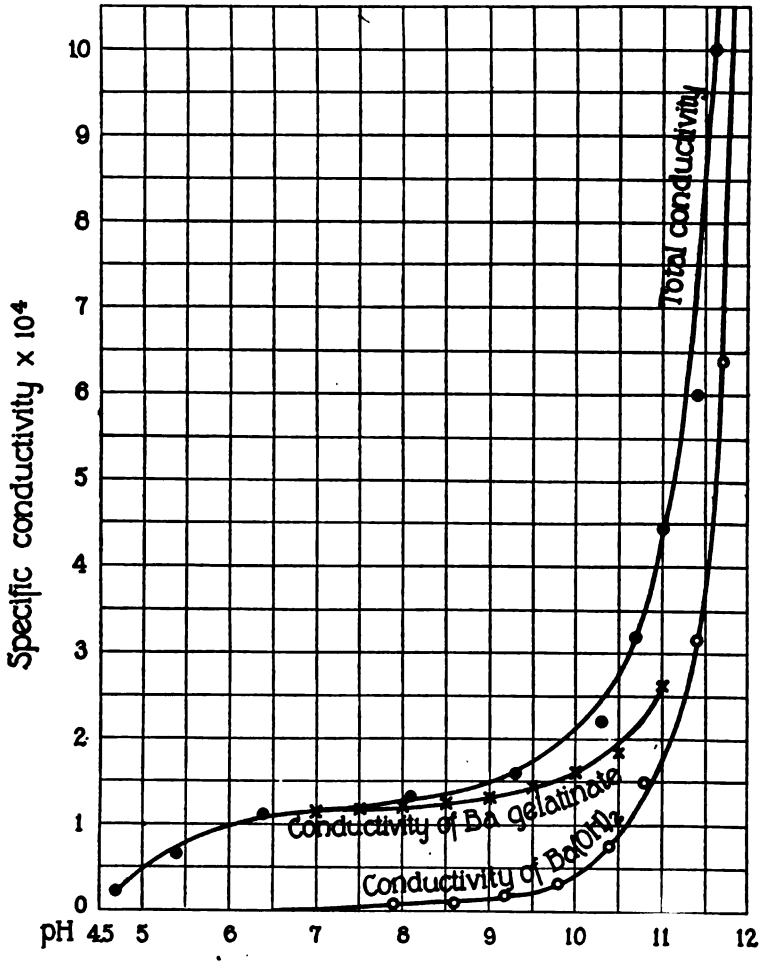

FIG. 9. Conductivity curve for Ba gelatinate.

bromide and gelatin sulfate is too small to account for the difference in the osmotic pressure of solutions of these two types of gelatin salts on the basis of differences in the ionization of the two protein salts.[9]

[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 569.

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These data lend no support to the assumption that the difference between the swelling, viscosity, and osmotic pressure of gelatin sulfate on the one hand, and of gelatin chloride and gelatin oxalate on the other is due to differences in the degree of ionization of proteins.

TABLE VI.

Specific Conductivity of 1 per cent Solutions of Gelatin Chloride, Gelatin Sulfate, and Gelatin Oxalate.

	pH of gelatin-acid salt.				
	4.4	4.0	3.7	3.4	3.0
Gelatin oxalate	0.65	1.45	2.15	3.15	5.25
" sulfate	0.75	1.75	2.60	3.95	6.15
" chloride	0.8	2.0	3.25	4.85	8.5

SUMMARY AND CONCLUSIONS.

1. Our results show clearly that the Hofmeister series is not the correct expression of the relative effect of ions on the swelling of gelatin, and that it is not true that chlorides, bromides, and nitrates have "hydrating," and acetates, tartrates, citrates, and phosphates "dehydrating," effects. If the pH of the gelatin is taken into considertion, it is found that for the same pH the effect on swelling is the same for gelatin chloride, nitrate, trichloracetate, tartrate, succinate, oxalate, citrate, and phosphate, while the swelling is considerably less for gelatin sulfate. This is exactly what we should expect on the basis of the combining ratios of the corresponding acids with gelatin since the weak dibasic and tribasic acids combine with gelatin in molecular proportions while the strong dibasic acid H_2SO_4 combines with gelatin in equivalent proportions. In the case of the weak dibasic acids he anion in combination with gelatin is therefore monovalent and in the case of the strong H_2SO_4 it is bivalent. Hence it is only the valency and not the nature of the ion in combination with gelatin which affects the degree of swelling.

2. This is corroborated in the experiments with alkalies which show that LiOH, NaOH, KOH, and NH_4OH cause the same degree of swelling at the same pH of the gelatin solution and that this swell-

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ing is considerably higher than that caused by $Ca(OH)_2$ and $Ba(OH)_2$ for the same pH. This agrees with the results of the titration experiments which prove that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent proportions and that hence the cation in combination with the gelatin salt with these two latter bases is bivalent.

3. The fact that proteins combine with acids and alkalies on the basis of the forces of primary valency is therefore not only in full agreement with the influence of ions on the physical properties of proteins but allows us to predict this influence qualitatively and quantitatively.

4. What has been stated in regard to the influence of ions on the swelling of the different gelatin salts is also true in regard to the influence of ions on the relative solubility of gelatin in alcohol-water mixtures.

5. Conductivity measurements of solutions of gelatin salts do not support the theory that the drop in the curves for swelling, osmotic pressure, or viscosity, which occurs at a pH 3.3 or a little less, is due to a drop in the concentration of ionized protein in the solution; nor do they suggest that the difference between the physical properties of gelatin sulfate and gelatin chloride is due to differences in the degree of ionization of these two salts.

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NUMERICAL LAW OF REGRESSION OF CERTAIN SECONDARY SEX CHARACTERS.

By A. PÉZARD.

(*From the Laboratory of General Biology of the College of France, Paris.*)

CORRECTION.

On page 150, Vol. iii, No. 2, November 20, 1920, line 18, for *The resistance after, etc.*, read *The resistance due to A and M after, etc.*; line 20, of the same page, equation (4) should read as follows:

$$\text{Resistance} = 2{,}475.22\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 70.69\left(e^{-K_M T}\right) + 10 \quad (4)$$

page 151, line 11, equation (5) should read as follows:

$$O = 89.1\left(\frac{K_N}{K_O - K_N}\right)\left(e^{-K_N T} - e^{-K_O T}\right) - 90\, e^{-K_O T} \quad (5)$$

line 16, of the same page, for *the value of O is 92.57* read *the value of O is 82.57*; lines 16 and 17, of the same page, for $(92.57 - 10) \div (100 - 10) = 0.917$ read $(82.57) \div (90) = 0.917$.

of a few weeks (5 to 12) the comb and similar organs reach a stable condition in which they present the reduced size and farinaceous aspect which characterize those of the castrated fowl of our first series. This result demonstrates conclusively that the internal secretion of

[1] Pézard, A., Le conditionnement physiologique des caractères sexuels secondaires chez les Oiseaux, *Bull. biol. France et Belg.*, 1918, lii, 1.

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NUMERICAL LAW OF REGRESSION OF CERTAIN SECONDARY SEX CHARACTERS.

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(From the Laboratory of General Biology of the College of France, Paris.)

(Received for publication, October 22, 1920.)

Postpuberal Castration.

When a young cock is deprived of its genital glands (prepuberal castration), it is found that the fleshy appendices which the head bears (Fig. 1), *i.e.* the comb, barbules, and "oreillons," do not develop, or that at least they follow the general law of growth of the body; moreover, instead of acquiring the brilliant color which they have in the normal adult cock, they remain pale and are covered with a fine, white film due to epithelial desquamation (farinaceous appearance).[1] Since these organs attain complete development when minute fragments of testicle are inserted into the peritoneum of castrated fowl, or when frequent injections of fresh testicular extract are given, we conclude that they are related to an internal secretion produced by the male genital glands. The question arises whether, once the comb and similar organs have developed, the testicle is still necessary to assure their maintenance.

To determine this point we have castrated not only cockerels but also adult cocks in full possession of their secondary sex characters. Regression of the comb begins during the first days following operation (Fig. 2). It is at first rapid but gradually diminishes until at the end of a few weeks (5 to 12) the comb and similar organs reach a stable condition in which they present the reduced size and farinaceous aspect which characterize those of the castrated fowl of our first series. This result demonstrates conclusively that the internal secretion of

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the testicle is just as necessary for the maintenance of these organs as it is for their growth.

In these experiments on postpuberal castration, the length and width of the comb were measured each week during the period of

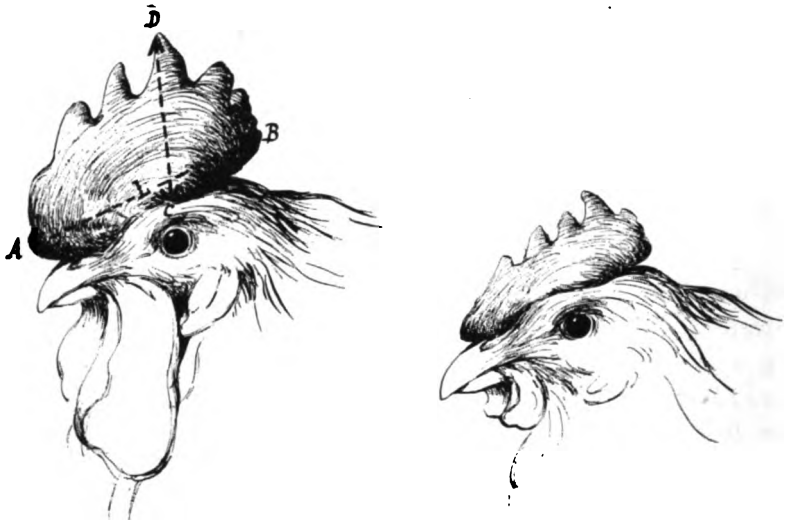

FIG. 1. Head of a normal adult cock (left) and head of a castrated cock (right).

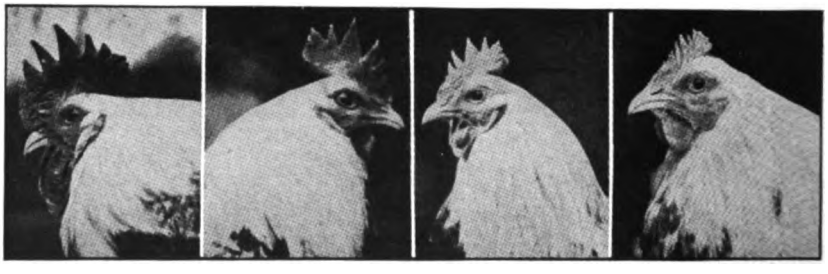

FIG. 2. Successive aspects of the comb of a cock during regression: (1) at the time of castration; (2) 5 weeks later; (3) 7 weeks later; and (4) at the end of regression.

regression. The length (Fig. 1) was measured from the anterior extremity (A) to the most distant point of the posterior portion (B); the width from the posterior point of origin (C) to the edge of the highest point of the crest (D). In this way the measurements are

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always relative to the same segments; the dimensions are given in millimeters. It was difficult to secure greater precision than this, nor would that inform us except on the secondary variations not susceptible of modifying the general appearance of the phenomenon. It should be noted, however, that the dimensions relating to length are relatively more precise than those having to do with width, since in the former case we were dealing with a larger figure.

A curve has been plotted representing the variation in length of the comb during regression. This curve may also represent the variation in volume, for the three dimensions were found to be reduced proportionately, as shown by careful observation of the organ in process of regression, which visibly retains the same form during the period of its etiolation. This observation may, moreover, be mathematically verified by measuring the linear dimensions and the weight of the comb (the latter being proportionate to the volume) in normal and in castrated males. The following is an example.

Nov., 1919. Normal cock. Comb, 73.40 mm. Weight, 8.8 gm.
Castrated " " 54.28 " " 3.1 "

If the proportions of the comb remained the same, the weight should be proportionate to the cubes of the homologous dimensions. This is found to be true.[2]

$$\left(\frac{73}{54}\right)^3 = 2.45 \left(\frac{40}{28}\right)^3 = 2.91 \frac{8.8}{3.1} = 2.84$$

We encountered a difficulty in determining the time of regression. Regression, at least when it follows postpuberal castration, occurs immediately after the operation (indicated on the figures as *Op*). On the other hand, the end of the regressive period is somewhat uncertain. In our experiments we have arbitrarily considered the regression as terminated when the comb undergoes oscillations of no more than a millimeter per week.

[2] This does not mean that the equation between the length of the comb and the time is exactly that between the volume and the time; nevertheless, it is easy to pass from one to the other.

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Results.

In the following paragraphs we shall indicate in each case: (1) information relating to the bird under observation; (2) a graphic chart on which are shown the observed curve and the parabola which most nearly approximates it; the latter departs from the horizontal at the point where regression begins and becomes tangent to the horizontal at the point where regression ends. This parabola may be represented algebraically by the formula

$$L = l + \tfrac{1}{2}C(\theta - t)^2$$

in which L represents the length of the comb at a given time during regression, l the length of the comb at the end of regression, C an individual constant, θ the total period of regression, and t the time corresponding to the unknown ordinate L.

Experiment 1.—Cock, Dorking, about 1 year old. Sex characters well developed; combs and barbules scarlet; sexual instincts well developed.

Castrated Mar. 20, 1912. Both testicles very large, total weight 42 gm. Regression of the comb began at once and was finished at the end of 12 weeks (Fig. 3). There was coincidence between the observed curve and the parabola throughout almost the entire length of the curve: only at Point 6 of the ordinate was there a deviation, regression having been accelerated by a cause unknown to us; after that the acceleration was compensated for, since the phenomenon resumed its normal curve.

Experiment 2:—Cock, Beauceronne, about 6 months old. Secondary sex characters in course of development but not completely developed. Comb brilliant but not yet of its final length. Completely castrated Jan. 18, 1912. Regression began immediately and continued for 7 weeks (Fig. 4.) There was the best possible coincidence throughout the entire extent of the curve.

The weight curve (shown by the broken line, the scale of weight being found at the right) indicates clearly that the curves are independent of each other and that the phenomenon of regression follows its regular course irrespective of the general nutrition of the bird. Regnault and then Pflüger later have shown that the body regulates its consumption not according to the substances which are offered it but according to the demands of the cells. Here we see clearly that the needs of the cells are in their turn influenced by the testicles, and that their nutrition is independent of the general metabolism.

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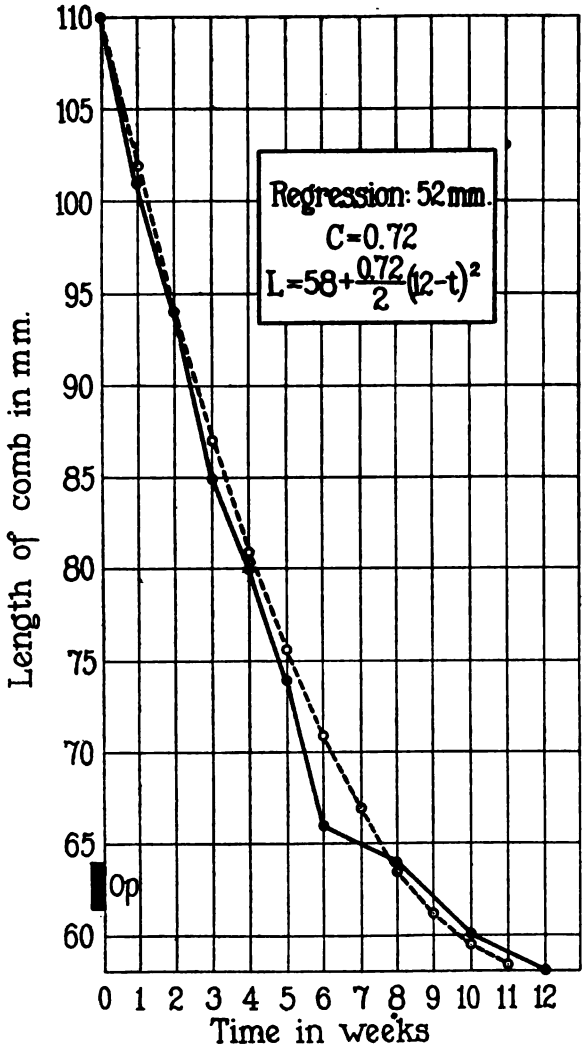

FIG. 3.

Experiment 3.—Cock, Black Orpington, about 8 months old. Castrated Dec. 4, 1909. The bird, whose sexual growth was slow, did not yet possess its secondary sex characters. It received from Jan. 17 to May 23, 1910, frequently repeated injections of testicular extract of the undescended testicle of a pig. Under this influence the comb developed and reached its normal length (86 mm.).

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On May 23 the injections were discontinued, and on May 26 the comb began its regression, which ended in July (Fig. 5).

Coincidence is particularly remarkable in the present case. Note that the last injection had been given May 23; regression did not actu-

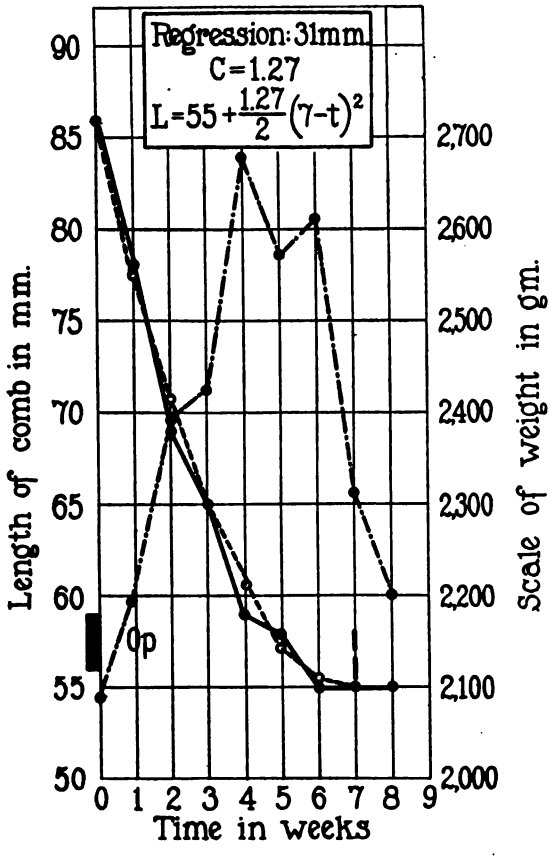

FIG. 4.

ally begin until May 26. This retardation is explained, in our opinion, by the slowness of absorption of the product introduced into the body intraperitoneally, owing possibly to the colloidal nature of this product.

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Experiment 4.—Cock, Faverolle, hatched July 1, 1913, began to display the secondary sex characters in Nov., notwithstanding an attempt at castration later recognized as incomplete. Two testicular nodules about the size of a hazelnut were extirpated Feb. 26, 1914; regression began immediately; we shall consider it as ending at 6 weeks (Fig. 6). Coincidence was not so good as in the other cases, for reasons which we are not in a position to explain at present; it should be added that during the 3 months following (May to July) regression continued but was negligible, being only 3 mm.

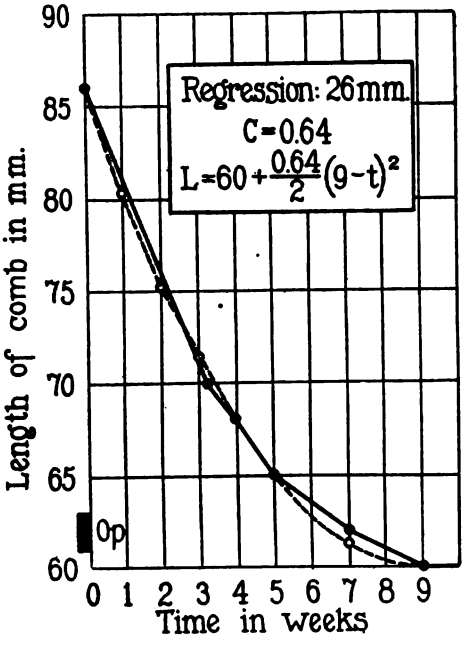

FIG. 5.

Experiment 5.—Cock, Bare-neck, hatched July, 1912. Castrated Nov. 11, 1912. Received in right peritoneal cavity fragments of ovary from a pullet of the same brood. These fragments did not appear at once to have any influence on the regression of the comb, which was effected at the normal rate. On the other hand, a small testicular nodule developed after the operation, and from the 7th week the phenomenon was reversed and a new growth of the comb brought about, hence the second branch of the curve. At autopsy this nodule was extirpated: the weight was 1.3 gm. It had the usual testicular structure. Although the two segments of the curve do not appear to correspond, the regression was complete (Fig. 7).

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The coincidence between the recorded and the theoretical curves is good in the lower points, and less satisfactory at the time corresponding to Points 2 and 3, when there was an acceleration of regression. The portion of the curve AB corresponds to the regression, the segment BC to the renewed development of the comb under the influence of the nodule of regeneration.

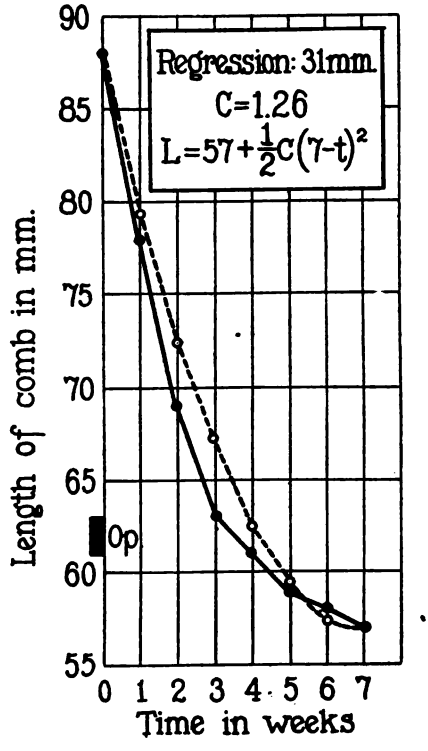

FIG. 6.

We have also plotted the weight curve, as in Experiment 2; there is the same independence as previously concerning the evolution of the comb and the general nutrition.

Comparison of the theoretical and the observed curves shows the coincidence to be as good as possible. Furthermore, if the curve represents the facts as exactly as possible, it must make extrapolation possible; that is, if we know, in a given instance, a portion of the curve

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sufficient for determining its constants, we must be able to calculate the other points of the curve beyond those derived from our experimental data. The calculation made on the basis of the measurements on Cock 6 (which died before regression was complete) leads us to conclude that such is the case.

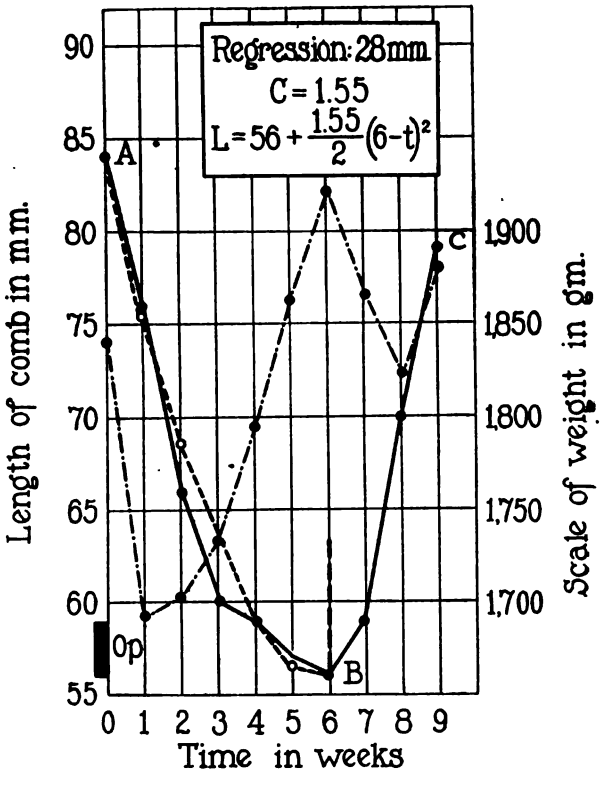

Fig. 7.

Experiment 6.—Cock, Beauceronne, about 6 months old. Secondary sex characters in course of development. Comb and barbules scarlet. Sex insticts present; crowing. Completely castrated Jan. 18, 1912; died Feb. 18 in a state of cachexia (Fig. 8).

Having made five measurements, we know five points of the curve; three are sufficient for the calculation of l, C, θ. Utilizing these, which correspond to 0, 1, and 4 weeks, we find:

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$$l = 56.5$$
$$C = 0.5$$
$$\theta = 10.5$$

The value found for l in this case is the same as that furnished by the subjects of the other experiments; the duration of regression is a little long, but not improbable.

The individual constant C is a measure of the negative acceleration of the regression. In order to determine to what factor it corre-

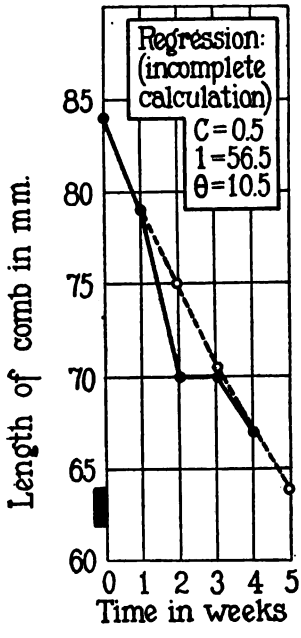

FIG. 8.

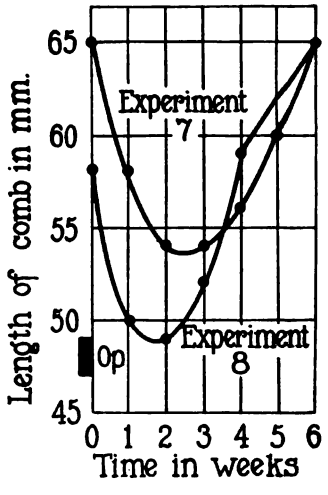

FIG. 9.

sponds, let us note that the abrupt beginning of the curve indicates that the action of the testicle does not survive castration. Under these conditions, the constant C appears to us to be related exclusively to the properties of the erectile tissue. For this reason we have called it the *constant of regression*. On its value and on that of the total decline depends the duration of regression.

Values found.						
C.....	1.55	1.27	1.26	0.72	0.64	0.5
θ.....	6.0	7.0	7.0	12.0	9.0	10 5

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It would be interesting to determine the variation with age of the total decline, the constant of regression C, and also the time θ. It is possible that C diminishes with age, while θ increases. But the number of experiments at our disposal do not permit us to affirm it. At all events, by the same method of reasoning, we may regard as established from now on the fact that C and θ are bound to each other by a simple relation. To demonstrate this point, we shall base our reasoning on the figures derived from our experiments on castration followed by transplantation.

Intrapuberal Castration.

The following observations have no bearing on the cocks which had acquired or nearly acquired their secondary sex characters, but upon those birds which were castrated at the time when the development of these characters was beginning (intrapuberal castration). As the diminution in the comb is then slight, one might expect to find a slow regression similar to that which characterized the end of the regression period in the previously observed birds the experiment confirms this hypothesis.

Experiment 7.—Cock, mixed breed, hatched at the Physiological Station in Aug., 1911. Castrated Jan. 22, 1912. Received testicular transplantations at the time of castration. For 3 weeks the comb regressed; then its development recommenced under the influence of the transplantations. The regression and even the resumption of development have a clearly parabolic curve (Fig. 9).

We had calculated C, θ, and l starting from the formula

$$L = l + \tfrac{1}{2}C(\theta - t)^2$$

obtaining successively

$$t = \begin{cases} 0 \\ 2 \\ 4 \end{cases} \qquad L = \begin{cases} 65 \\ 54 \\ 56 \end{cases}$$

whence

$$C = 3.25$$
$$\theta = 2.7$$
$$l = 53.2$$

Experiment 8.—Cock, same conditions of experiment and same succession of observations as in the preceding case (Fig. 9). The calculations are the same as in Experiment 7, according to the general formula

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$$t = \begin{cases} 0 \\ 2 \\ 4 \end{cases} \qquad L = \begin{cases} 58 \\ 49 \\ 59 \end{cases}$$

whence

$$C = 4.75$$
$$\theta = 1.9$$
$$l = 48.9$$

In both cases, the regression due to intrapuberal castration began immediately after castration, as in normal subjects. Furthermore, although the mass of tissue before regression was much smaller, *the decline began with the same rapidity as in adults*: the diminution in length of the comb was 7 mm. (Experiment 7) and 8 mm. (Experiment 8) during the 1st week. That the length attained at the end of regression was much less than in the preceding cases is explained by the fact that the comb of a castrated fowl of the same age has not yet attained its final length. As to the duration of regression, it is much shorter than in the preceding experiments, now that the constant C is much smaller.

SUMMARY.

The discrepancy in the relative variation of C and of θ led us to examine more closely the velocity of regression at the beginning in all the cases. At a given point of the curve, the velocity is furnished by the differential quotient of the length with reference to the time:

$$v = \frac{dL}{dt} = -C\theta + Ct$$

At the beginning of regression, that is to say, at the time 0

$$v_0 = -C\theta$$

We have tabulated the corresponding numerical values in the various instances:

Experiment No.	C	θ	$-C\theta$
8	4.75	1.95	−9.4
7	3.25	2.7	−8.7
5	1.55	6.0	−9.3
2	1.27	7.0	−8.9
4	1.26	7.0	−8.8
1	0.72	12.0	−8.6
3	0.64	9.0	−5.8 (Species with small comb.)
6	0.5	10.5	−5.2 (Extrapolation.)

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Although there is not absolute equality among the figures of the last column, one cannot fail to be struck by the fact that there is very little difference; in all instances they diverge much less than those of the first two columns, in which the variation is from 0.5 to 4.75 and from 1.95 to 12.0. We must admit, therefore, within rather wide limits, the constancy of the product of the time of regression and the constant C, whether the castration is intrapuberal or postpuberal.

Geometrically, this result is represented by the constancy of the angle of the ordinate and the tangent to the parabola at the point of departure of the regression curve. Furthermore, it follows that the numerical law is represented not only by a parabola, but more exactly by segments of homothetic parabolas—an unexpected generalization, which gives a remarkable unity to the law with which it is concerned.

CONCLUSIONS.

1. Postpuberal castration is followed in cocks by a regression of the comb; this organ acquires in a few weeks the aspect and dimensions which characterize similar organs of cocks castrated before puberty.

2. The diminution in the length of the comb is effected according to a simple law, represented geometrically by a segment of a parabola, algebraically by the equation

$$L = l + \tfrac{1}{3}C(\theta - t)^3$$

3. Intrapuberal castration demonstrates that during the development of the comb C and θ have values very different from those which are furnished by adults, but the product $C\theta$ is constant.

4. This constancy indicates that the diverse curves are represented by similar segments of a parabola.

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PHOTOCHEMISTRY OF VISUAL PURPLE.

II. The Effect of Temperature on the Bleaching of Visual Purple by Light.

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(Received for publication, September 23, 1920.)

I.

1. The determination of the influence of temperature on the velocity of a photochemical reaction is always of importance. This is particularly true when the mechanism of the reaction is unknown, as in the present instance of the bleaching of visual purple.

The usefulness of determining the temperature coefficient lies in this. Photochemical reactions have low temperature coefficients,—near unity (Sheppard, p. 304), though exceptions are known. Ordinary chemical reactions, however, have temperature coefficients which are much higher, between 2 and 3 or even more. The order of magnitude of the temperature coefficient may therefore give us some notion of the degree of complexity of a photosensitive reaction. A value of Q_{10} near unity may be taken to indicate that the reaction probably consists of a simple, direct photochemical transformation. On the other hand, a high value for Q_{10} shows most probably that the photochemical reaction proper is only one in a series of reactions which makes up the photosensitive process. The associated chemical reactions, because of their high temperature coefficients, raise the temperature coefficient of the reaction as a whole more nearly to their own level.

An example of just such a condition is the high temperature coefficient (Q_{10} = 2.5) of the photosensory process in *Mya*. On analysis (Hecht, 1918–19, *a*; 1919–20, *b*) a series of at least three reactions has been shown to exist, of which the photochemical proper is only the first. This has a temperature coefficient of 1.06 when investi-

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gated independently; the values for the temperature coefficients of the other reactions are 2.5 (μ = 19,680) and 13.5 (μ = 48,500) respectively. An analysis of a similar hypothetical condition has been made by Osterhout (1917) which shows the relation of the total temperature coefficient of a process to the temperature coefficients of the individual reactions composing it.

2. In the first paper of this series (Hecht, 1920–21, *c*) it was shown that visual purple solution, so prepared as to give an irreversible photochemical reaction, is bleached by light according to the kinetics of a monomolecular reaction. This in itself would seem to indicate that the reaction as a whole is a simple photochemical transformation.

Experiments with intermittent as compared with constant illumination supported this conclusion. Within the limits of experimental error it was not possible to demonstrate the presence of any initial induction period or of an after effect in the course of the reaction. The presence of either or both of these irregularities frequently bespeaks a complexity in the make-up of the reaction (Mellor, 1904, p. 116).

The evidence therefore indicates that the bleaching of visual purple is a comparatively simple process. This is further confirmed by the following experiments on the effect of temperature.

II.

Visual purple solutions are prepared from the dark-adapted retinas of frogs by the technique previously described.[1] The changes produced by light are followed colorimetrically by comparison with concentration standards. These are made by combining bleached and unbleached visual purple in varying proportions so as to give a series changing by 10 per cent steps. The experimental solutions and the standards are manipulated in capillary tubes arranged to give equal depths of solution. For the composition and preparation of the standards and for the methods generally, the reader is referred to the previous article in these studies (Hecht, 1920–21, *c*) where full details will be found.

[1] In making solutions of visual purple, pure bile salts must be used. I am indebted to Dr. S. Morgulis for furnishing me with a supply of these salts purified by himself.

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In order to control the temperature of the exposed solution the capillary tube containing it is kept in a water bath. This is a cylindrical glass vessel, 12 cm. in diameter, and of 900 cc. capacity. The temperature of the bath is maintained constant by the addition of water at a higher or a lower temperature from a nearby reservoir. In this way the temperature is easily kept within 1°C. Such a bath is accurate enough for our purposes, as will presently be evident.

The exposure tube of visual purple is immersed, attached to the rotating apparatus, and kept in the dark for 5 minutes for its contents to reach the temperature of the bath. This is more than enough when we recall that a thermometer having a larger diameter than the capillary tube comes into thermal equilibrium in much less time. After 5 minutes the shutter is opened, and the light allowed to act on the solution, which is rotated in the usual manner. After the proper exposure, the light is shut off, and the concentration of the exposed solution determined. As in the previously reported work, readings are made to the nearest 5 per cent. The tube is then returned to the bath, allowed 5 minutes in the dark to come to the temperature of the bath, and again exposed for an interval. The concentration is again determined, and the process repeated until the solution is fully bleached.

The source of illumination is a 250 watt, concentrated-filament, stereopticon Mazda lamp run on the ordinary lighting circuit. The intensity used throughout these experiments on temperature effects is 50 meter candles. At this intensity bleaching is complete in half an hour.

III.

A number of preliminary experiments showed definitely that the effect of temperature on the velocity of the bleaching process is very small indeed. In this I can confirm the roughly quantitative, early work of Kühne (1879). For example, in a series of three experiments performed, one at 7°C., another at 23°C., and a third at 36°C., the velocity constants for the bleaching process were 0.038, 0.041, and 0.039 respectively. This gives a value for the temperature coefficient as $Q_{10} = 1.00$. The same value for Q_{10} was found for a set of experiments in which the velocity of decomposition was determined

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at five temperatures covering the same interval as the previous three. However, in two other series of experiments performed at three temperatures as before, I obtained a value for $Q_{10} = 1.15$.

These four sets of preliminary experiments were carried out by the use of a single tube of visual purple for each temperature. Individual experimental errors may thus possibly account for the differences between the first two and the last two sets of experiments. Therefore, in order to be thoroughly certain of the results, I performed two sets of experiments in which three tubes of visual purple

TABLE I.

Temperature and Bleaching of Visual Purple.

Temperature.	$k=\frac{1}{t}\log\frac{a}{a-x}$	
	Series 1.	Series 2.
°C.		
5.2	0.039	0.031
20.0	0.035	0.031
36.1	0.036	0.033

were bleached simultaneously at each temperature. The velocity constants for the two series are given in Table I together with the temperatures. Each figure is the average of three experiments which agreed among themselves in a way similar to the figures previously quoted for one preliminary experiment.

IV.

1. From these results it will be seen that the change in the velocity constant over a range of 30 degrees is practically *nil*. Graphically this is demonstrated by Fig. 1, which presents the details of the second series of Table I. The rectangles are the averages of the experimental findings at the different temperatures, whereas the curve is the isotherm of a monomolecular reaction

$$k = \frac{1}{t}\log\frac{a}{a-x}$$

in which the velocity constant has the average value of $k = 0.032$. It is apparent that the points at all temperatures fit the single curve as well as can be expected.

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It may therefore be concluded that the temperature coefficient for 10°C. for the bleaching of visual purple by light is practically 1.00. This corroborates our idea that the bleaching reaction is a simple photochemical transformation, consisting most probably of a single reaction.

2. There is an additional point to be made with regard to the magnitude of the temperature coefficient. In any photochemical

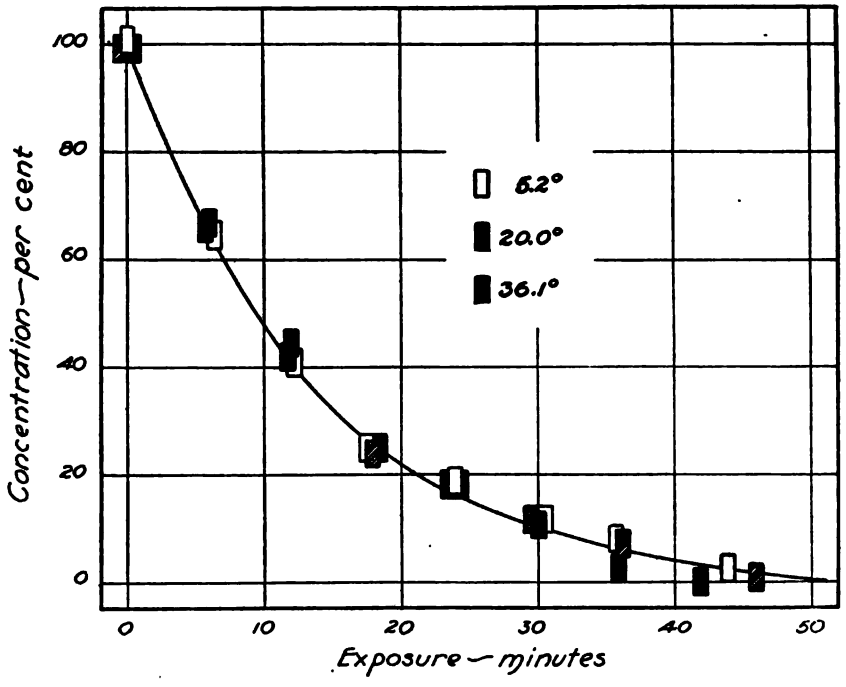

FIG. 1. Temperature and the velocity of photochemical decomposition of visual purple. The curve is a monomolecular isotherm; the rectangles are the experimental results of one set of experiments.

reaction there is always the possibility that the reaction proper takes place with a practically instantaneous velocity at the walls of the vessel, and that the kinetics of the reaction really represent the diffusion velocity of the photosensitive substance to the place of reaction.

The temperature coefficient of diffusion velocities, though lower than those of ordinary chemical reactions, is usually higher than 1.00,

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—generally near 1.3 (Höber, 1914, p. 708). The fact that the temperature coefficient of visual purple bleaching is 1.00, combined with the other evidence already at hand, would therefore indicate that the kinetics of the reaction represent a real chemical occurrence rather than a process of diffusion.

SUMMARY.

The temperature coefficient of the bleaching of visual purple by light is 1.00 over a range of 30 degrees. This indicates that the monomolecular course of the reaction represents a real chemical process, as opposed to a possible diffusion process, and that the reaction is probably simple in nature.

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THE AMPHOTERIC PROPERTIES OF SOME AMINO-ACIDS AND PEPTIDES.

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(Received for publication, October 27, 1920.)

INTRODUCTION.

The change in the hydrogen ion concentration of a solution upon the addition of acid or alkali has been used to follow the changes characteristic of certain dissolved substances. Thus, the progressive neutralization of polybasic acids and of polyacid bases has been studied in this way.[1] Amphoteric electrolytes lend themselves readily to this method of investigation. Recently, the results obtained with a number of proteins have been presented in the form of titration curves in which amounts of standard acid and alkali were plotted along one axis and the resulting pH values of the protein solution along the other axis.[2] Such titration curves may be expected to be more or less characteristic for the acid- and base-combining properties or groups of such substances. In view of the importance and value of such studies in protein chemistry, the titration curves of some of the simple amino-acids and dipeptides are presented here as a necessary preliminary to a more satisfactory understanding of the more complex bodies.

Experimental Methods.

The following substances were studied; glycine, alanine, α-amino-butyric acid, leucine, glycyl-glycine, alanyl-glycine, and alanyl-alanine. Data were obtained also for solutions of sodium chloride, acetone,

[1] Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 847.

[2] *Cf.* Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145. Cohn, E. J., Wolbach, S. B., Henderson, L. J., and Cathcart, P. H., *J. Gen. Physiol.*, 1918–19, i, 221. Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1915–17, xii. Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, especially pp. 34 and 211, for general discussion and references.

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acetamide, urea, acetic acid, and aceturic acid. The substances were prepared by the usual methods and showed satisfactory states of purity except for the alanyl-alanine, for which the nitrogen content was found to be 1 per cent less than the calculated value. Attempts at purification did not remedy this, so that evidently an impurity, possibly alanine, was present.

The solutions studied were prepared so that 1 liter of the final mixture contained in every case 0.05 gram molecule of the amino-acid or peptide or other substance, and 0.05 gram molecule of sodium chloride, while the amounts of hydrochloric acid or sodium hydroxide (0.1 N) added were varied. The water used was distilled first from sulfuric acid, then from alkaline permanganate, and condensed in a block tin condenser.

The concentration of the ampholyte or other substance and of the sodium chloride in the final volume (50 cc. as made up and used) was the same in all cases. The only variable was the amount of acid or alkali which was added, or the ratio between this and the concentration of solute.

The hydrogen ion concentrations of the solutions were determined by electromotive force measurements using calomel electrodes with 0.1 M potassium chloride solution, saturated potassium chloride bridge, unknown solution with hydrogen electrode (platinum or gold coated with palladium black), and a potentiometer of Leeds and Northrup type "K" in conjunction with a type "H" galvanometer with a loading coil to decrease its period. Cells of the rocking type described by Clark[3] were used with the modification of having an adapter of about 25 cc. capacity fused on the inlet tube.[4] Hydrogen was obtained compressed in cylinders and was purified by passage through (electrically) heated copper gauze and then bubbled through water.

The customary precautions in purifying the materials, and standardizing the various parts of the apparatus and the solutions were taken. The hydrogen ion concentrations in terms of pH were calculated from the experimental measurements by the formula[5]

[3] Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.

[4] The writers desire to thank Professor F. S. Lee of the Department of Physiology of Columbia University for presenting to them the excellent rocking apparatus used in this work.

[5] Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1909, viii, 22, 29.

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$$pH = \frac{\pi - 0.3377}{0.0577 + ((t - 18)\,0.0002)}$$

in which π = reading in volts; 0.3377 = potential of calomel electrode; and $0.0577 + ((t - 18)0.0002)$ = correction factor for temperature $t°$, as a thermostat was not used.

In a number of cases the hydrogen ion concentrations were also determined colorimetrically. Where this was done, the indicators and standard solutions described by Clark and Lubs were used.[6]

The isoelectric points of the various ampholytes were also determined by the method described by Michaelis[7] and developed by Sörensen.[8] This consists of adding the pure ampholyte solution to a series of solutions of progressively changing hydrogen ion concentration containing the indicator and noting where no change in color occurred on mixing the unknown ampholyte solution and standard solution.

Experimental Results.

The results obtained for the pH values for the solutions of the various substances in the presence of acid and alkali are given in Table I. Although not stated in the table, it may be recalled that all the solutions contained sodium chloride of the concentration 0.05 M. Column 1 headed "Water" contained no added solute but the sodium chloride and acid or alkali. The headings of the remaining columns indicate the solute present, 0.05 M in every case.

The titration curves plotted from these results are given (Figs. 1, 2, and 3). The pH values are given as abscissæ, and as ordinates are shown the number of cc. of 0.1 N hydrochloric acid or sodium hydroxide added to 25 cc. of 0.1 M solution of the indicated solute (also 0.1 M with respect to sodium chloride), and the whole diluted to 50 cc. The numbering of the curves corresponds to the numbering of the columns in Table I. Since the results of the amino-acids are so nearly alike, only one curve (Curve 6, Fig. 2) is given. Similarly for the dipeptides, only one curve (Curve 10, Fig. 3) is presented.

[6] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191.

[7] Michaelis, L., *Biochem. Z.*, 1912, xlvii, 251. Hasselbalch, K. A., *Biochem. Z.*, 1916, lxxviii, 129.

[8] Sörensen,[3] p. 150.

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TABLE I.

Milliequivalents of HCl or NaOH per liter of solution containing 50 milliequivalents of solute.	(1) Water.	(2) Urea.	(3) Acetamide.	(4) Acetone.	(5) Acetic acid.	(6) Glycine.	(7) Alanine.	(8) α-Aminobutyric acid.	(9) Leucine.	(10) Glycylglycine.	(11) Alanylglycine.	(12) Alanylalanine.	(13) Aceturic acid.
						HCl							
50	1.32	1.29	1.29	1.29	1.27	1.94	1.84	1.75	1.85	2.26	2.21	2.18	1.23
44						2.02	1.95	1.85	1.97	2.48			
40	1.45	1.39	1.39	1.39		2.11	2.05	1.97	2.05	2.65	2.74	2.55	
34						2.26	2.18	2.08	2.21	2.89	2.83		
30	1.55	1.50	1.50	1.50	1.49	2.32	2.32	2.25	2.30	3.00	2.97	2.75	1.46
24						2.51	2.49	2.42	2.44	3.29	3.15	3.09	
20	1.68	1.68	1.69	1.67		2.62	2.61	2.54	2.55	3.34	3.28	3.21	
18						2.70	2.68	2.62	2.63	3.42	3.39		
14	1.85					2.85	2.82	2.76	2.77	3.58	3.53	3.59	
10	2.07	1.97	2.00	1.97	1.95	3.02	3.01	2.92	2.94	3.75	3.71	3.83	1.88
8						3.13	3.11	3.04	3.07	3.88	3.84	3.90	
6	2.23				2.09	3.28	3.25	3.16	3.21	4.02	3.99	4.03	2.03
4						3.46	3.43	3.35	3.42	4.21	4.19	4.35	
2	2.71				2.52	3.75	3.72	3.63	3.71	4.65	4.42	4.58	2.24
1	2.96					4.06	4.04	3.92	4.05	4.82	4.74	5.16	

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NaOH													
0	4.31	4.82	5.00	4.31	2.88	5.39	5.34	4.75	6.12	5.64	5.21	5.59	2.36
1	10.31			10.63		8.04	8.02		7.89	6.58	6.43	6.48	2.39
2	10.98			11.05	3.25	8.34	8.35	(7.14)	8.19	6.93	6.76	6.84	2.49
4						8.70	8.71	8.48	8.53	7.24	7.10	7.17	
6	11.64			11.70	3.67	8.83	8.85	8.73	8.71	7.45	7.32	7.33	2.69
8						9.07	9.00	8.94	8.86	7.61	7.53	7.50	
10	11.90	11.92	11.88	11.93	3.93	9.16	9.13	9.10	9.01	7.71	7.59	7.61	2.90
14	12.04					9.34	9.31	9.32	9.19	7.88	7.83	7.80	
18						9.50	9.47	9.51	9.35	8.03	7.93	7.93	
20	12.20	12.23	12.21	12.27	4.34	9.55	9.54	9.57	9.43	8.11	8.01	8.01	3.28
24						9.71	9.78	9.73	9.56	8.26	8.23	8.16	
30	12.38	12.40	12.34	12.43	4.69	9.89	9.98	9.92	9.77	8.43	8.39	8.36	3.64
34						10.02	10.12	10.08	9.89	8.61	8.55	8.53	
40	12.50	12.51	12.45	12.59	5.10	10.28	10.37	10.27	10.12	8.85	8.75	8.77	4.12
44						10.49	10.58	10.46	10.35	9.08	9.05	9.08	4.50
50	12.59	12.54	12.65	12.64	6.51	10.86	10.93	10.88	10.81	9.78	9.81	10.23	(10.05)

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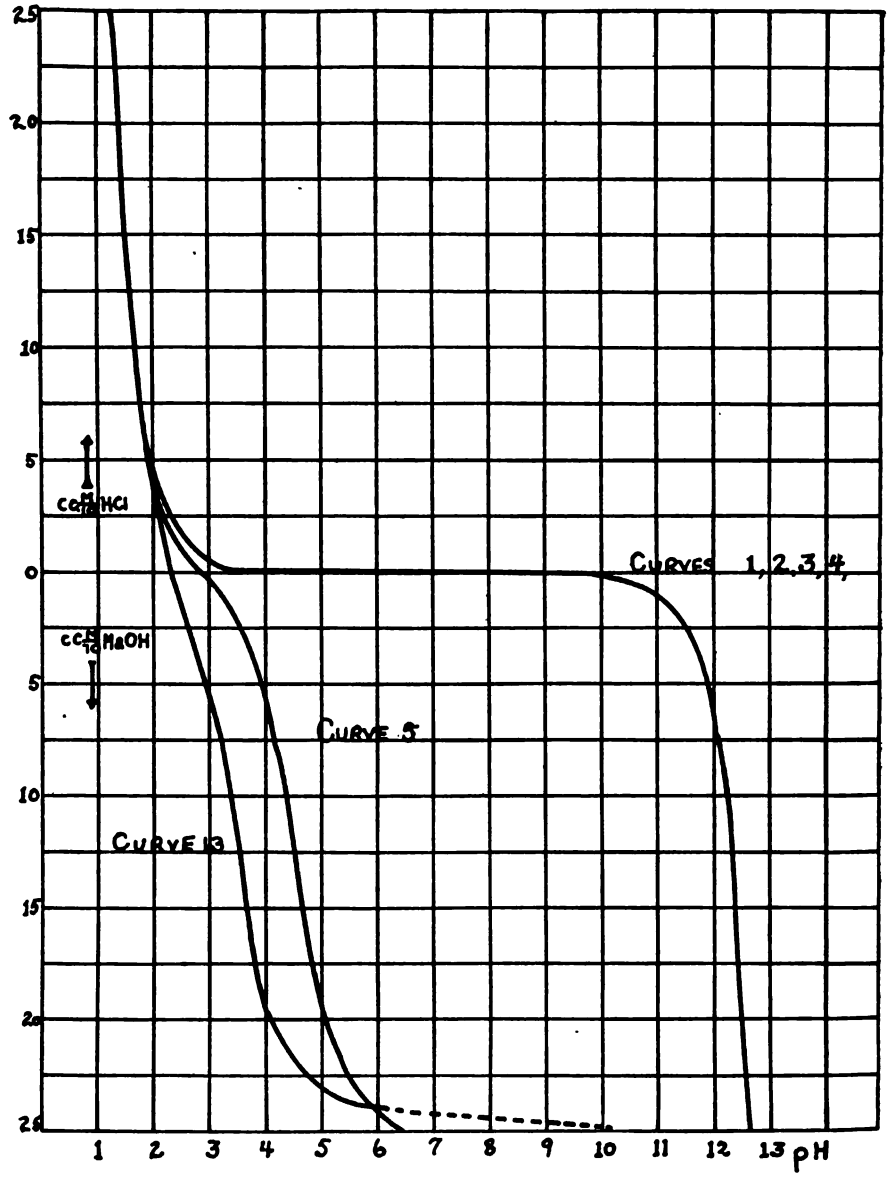

FIG. 1. Titration curves of water (1), urea (2), acetamide (3), acetone (4), acetic acid (5), and aceturic acid (13).

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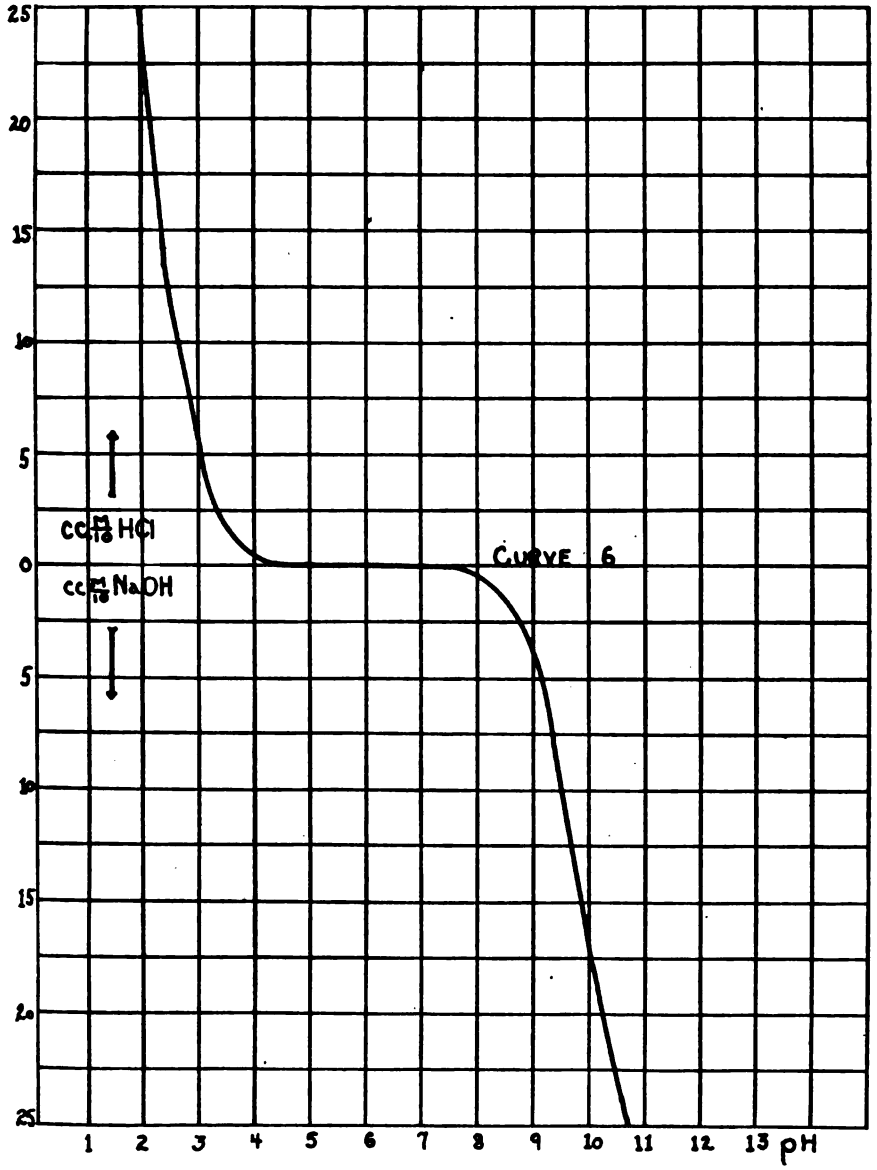

FIG. 2. Titration curve of glycine. Practically identical with titration curves of alanine, α-amino-butyric acid, and leucine.

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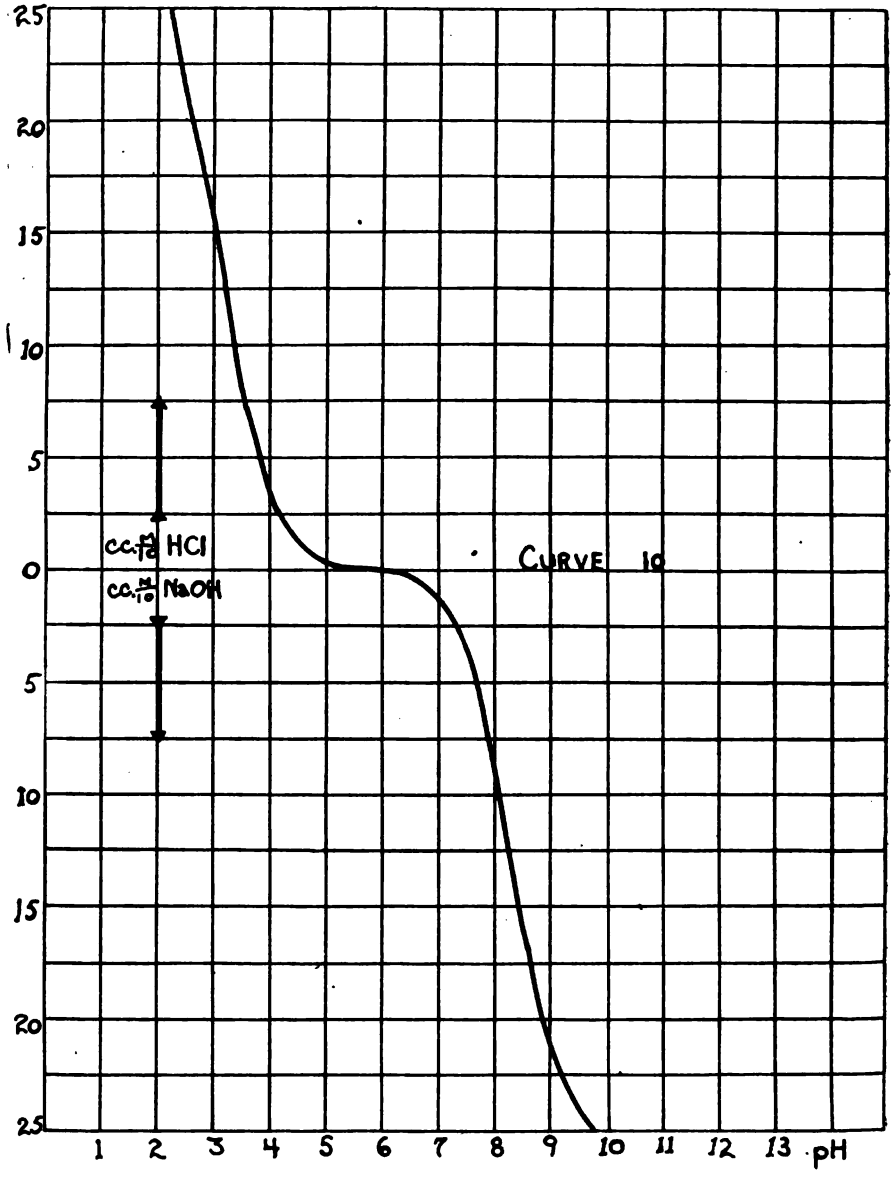

FIG. 3. Titration curve of glycyl-glycine. Practically identical with titration curves of alanyl-glycine and alanyl-alanine.

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The values for the isoelectric points of some of the substances are given in Table II. The significance of these points will be discussed in detail later in this paper. The values calculated by means of the expression

$$I = \sqrt{k_w \frac{k_a}{k_b}}$$

in which k_a and k_b represent the ionization constants of the ampholyte substance acting as an acid and as a base, and k_w the ionization constant of water (very nearly 10^{-14} at the temperatures used) are given and also the values determined experimentally by means of the potentiometer (values from Table I), and by the indicator methods.

TABLE II.

Isoelectric Points of Some Amino-Acids and Peptides.

	k_a*	k_b*	Isoelectric points.		
			Calculated.	Potentiometer.	Indicator.
			*p*H	*p*H	*p*H
Glycine	1.8×10^{-10}	2.7×10^{-12}	6.1	5.4	6.0
Alanine	1.9×10^{-10}	5.1×10^{-12}	6.2	5.3	6.15
Leucine	1.8×10^{-10}	2.5×10^{-12}	6.1	6.1	7.0
Glycyl-glycine	1.8×10^{-8}	2.0×10^{-11}	5.5	5.6	5.5
Alanyl-glycine	1.8×10^{-8}	2.0×10^{-11}	5.5	5.2	5.15
Alanyl-alanine				5.6	6.15

* Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546. Euler, H., *Z. physiol. Chem.*, 1907, li, 219.

Before discussing these results, a number of points connected with the experimental measurements will be taken up briefly; first for the results given in Table I, then for those in Table II.

Recently it was stated[9] that "There is hardly any use attempting the measurement of unbuffered solutions, if indeed there would be any significance to the measurement were it accurate." This statement apparently necessitates further explanation, possibly basing the definition of the term buffer in some way upon the slope of the titration curve. Otherwise, solutions of hydrochloric acid, etc., would be

[9] Clark,[2] p. 184. *Cf.* also a similar statement on p. 34.

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included among those for which accurate determinations are not possible.

This raises the question of possible inaccuracies in the determinations. Aside from such questions as the presence of small amounts of impurities at or near the isoelectric points of ampholyte solutions where the buffer action is found to be small,[10] certain points connected with the relative accuracies of the chemical and electrical measurements are involved. Comparing, for example, the determination of the hydrogen ion concentration of 0.1 N hydrochloric acid (pH approximately 1.0) and of 0.1 N sodium hydroxide (pH approximately 13.0), the electrical measurement is much more sensitive for the latter. The component determined in both cases is the hydrogen ion. In the preparation of the solutions, the alkaline solution is more readily influenced or changed (perhaps due to the accidental nature of the surroundings) as regards its hydrogen ion concentration. Alkaline solutions may therefore require the presence of buffer mixtures in order to obtain constant and reproducible results under conditions with which analogous acid solutions apparently give accurate values with no buffer present.

The presence of sodium chloride of the given concentration had no appreciable influence on the determinations as shown by the work of Harned[11] and of Fales and Nelson[12] on mixtures of acid and neutral salts, of Sörensen[13] who compared ampholyte solutions with and without salt, and of Tague[14] who measured amino-acid solutions without salt.

Some determinations by others may be compared with the results given here, although in most cases the results are not comparable for a whole series. The solutions with no added solute besides the acid and alkali agree satisfactorily with such results[15] except that Tague appeared to be more successful in excluding carbon dioxide. The results for glycine agreed well with those of Sörensen[13] and of Tague[14] where comparison is possible.

[10] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, for a careful and systematic discussion of these relations.

[11] Harned, H. S., *J. Am. Chem. Soc.*, 1915, xxxvii, 2460.

[12] Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2773.

[13] Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1909, viii.

[14] Tague, E. L., *J. Am. Chem. Soc.*, 1920, xlii, 173.

[15] *Cf.* Frary, F. C., and Nietz, A. H., *J. Am. Chem. Soc.*, 1915, xxxvii, 2264.

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The results have been presented without introducing the correction used by Tague in his work in which the amount of acid or alkali required to bring the solution without solute to a definite hydrogen ion concentration was subtracted from the amount required with the solute present. This corrected value would give a more nearly accurate measure of the reaction between amino-acid, etc., and acid or alkali. For the results given here, this correction is negligible except for the largest additions of acid or alkali. If desired, the data given in Column 1 of Table I will permit the introduction of these corrections.

With regard to the isoelectric point results given in Table II, the determinations by the potentiometer method, that is the determination of the hydrogen ion concentrations of the solutions with neither acid nor alkali added, cannot lay claim to any great degree of accuracy for the amino-acids since the buffer action is so slight and since a broad zone exists on the curve where traces of acid or alkali may change the results appreciably. With the dipeptides this zone is narrower and the results should be correspondingly more accurate. The indicator method appears to give somewhat more accurate results as fewer manipulations are required. However, too much stress must not be placed upon their exactness.

Discussion of Results.

The data given refer to one concentration (0.05 M) for each substance. This naturally limits for the present the general applicability of the conclusions.

Michaelis[10] and others[16] have developed the theoretical interpretation of such titration curves and applied their conclusions to the results of a number of substances. Among other applications, they have considered the relations to the acid and basic dissociation constants of the ampholyte, to conditions for the smallest and greatest buffer actions, etc. In this paper, the discussion will be limited to questions which have been discussed briefly or not at all by others.

[16] *Cf.* Clark[2] for the general treatment and for complete references.

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The following conclusions may be drawn from the results given in Table I and shown in the curves.

(*a*) The results for water, urea, acetamide, and acetone are practically identical. There is therefore at the dilutions used no combination involving the amino groups of urea or acetamide and hydrochloric acid, or enolization of urea, acetamide, or acetone accompanied by reaction with alkali which would be manifested by a change in hydrogen ion concentration.

(*b*) The results for the four amino-acids are nearly the same, showing only minor differences. Thus, on the acid side, alanine and leucine are identical, glycine and α-amino butyric acid differing slightly and in opposite directions; on the alkaline side glycine and alanine are the same differing slightly from the other two. There is a broad zone near the point at which no acid or alkali was added where practically no buffer action is shown.

(*c*) The results for the dipeptides, except for a few apparently minor differences, are strikingly similar throughout.

(*d*) As compared with the amino-acids, there was a very much smaller range where little or no buffer action was shown by the dipeptides. Aside from this, the curves for the two series were practically parallel. The vertical differences between the two sets of curves show the differences in the amounts of acid and of alkali required to bring the substances to the same pH values. Much more acid or alkali was required for the dipeptides than for the amino-acids, the difference being due evidently to the $-CO-NH-$ group of the dipeptides. The differences increase with increasing quantities of acid or alkali added, reach a maximum, and then decrease again. The chemical nature of the $-CO-NH-$ group readily accounts for these properties, acid combining with the $-CO-NH-$ group, alkali bringing about enol-lactim rearrangement and accompanying reaction.

(*e*) Aceturic acid on the acid addition side showed the same results as acetic acid and water. The influence of the highly ionized hydrochloric acid predominated over any effects of the weaker acids. On the alkaline side, the pH values found for aceturic acid were smaller than those for acetic acid with the same amount of alkali. This difference is evidently connected with the presence of the $-CO-NH-$ group and analogous to the difference between dipeptides and amino-

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acids. The vertical differences between the two curves also show the extent of base-combining property or enol-lactim tautomerism.

The method of plotting pH values against the amounts of acid and alkali added is also open to question, but it appears to be the best method which is available. Placing the emphasis on the hydrogen ion concentration gives a one-sided chemical perspective, especially for the alkaline solutions. The suggestion of Wherry[17] to use pH 7.0 as the zero point and to calculate values from this as a logarithmic function is not practicable as indicated by Clark.[18] The suggestion may be made to determine a zero point for each substance, this point to be identical with the isoelectric point, and to measure acidity and alkalinity from this point logarithmically in terms similar to the pH scale. This would fix a characteristic property for each substance as the starting point. The practical difficulty lies in the determination of the isoelectric point, as its experimental measurement is probably the least accurate point of most titration curves.

The general chemical nature of amphoteric electrolytes will be taken up briefly here. These substances, depending upon conditions, ionize or react as salts in which the complex ampholyte component acts as the positive constituent or as the negative constituent. Loeb[19] has recently pointed out clearly and convincingly, and has presented considerable experimental evidence to show, that the positive ampholyte ion (of gelatin, for example) is fundamentally different from the same ampholyte as negative ion. The relative acidity and alkalinity of the solution govern these changes, the ionization taking the different courses in more acid or more alkaline solutions (the transition point will be taken up presently). In order to account for the different processes of ionization, it appears to be necessary to assume that the molecule (unionized) is different in the two cases. In order to ionize so that the complex is part of the cation, the molecule must possess a structure different from that of the molecule which ionizes with the complex as part of the anion. This difference in structure may be due to an isomeric rearrangement, possibly tautomeric, to a difference in the action of the solvent involving hydration, or to some

[17] Wherry, E. T., *J. Washington Acad. Sc.*, 1919, ix, 305.

[18] Clark,[2] p. 28.

[19] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559; 1919–20, ii, 87.

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other cause. An equilibrium relation between the two (or more) unionized forms would exist as a rule. This conception complicates somewhat the theoretical treatment of amphoteric electrolytes presented by Bredig,[20] Walker,[21] and others by the addition of an equation representing such an equilibrium and by introducing the concentration of the suitable molecular species in the equations representing the ionization constants. In strongly acid or alkaline solution, however, practically only one form would be present.

The difference in the ionization of the ampholyte is not always brought out clearly. For example, with a protein, the two kinds of ions are sometimes indicated as Na^+ (Protein$^-$) and (Protein$^+$) Cl^-. As a matter of fact, rarely, if ever, do the ions (Protein$^-$) and (Protein$^+$) have even the same chemical composition. This may be indicated by taking the simple case of glycine. Here, the ions are $Cl^-(NH_3CH_2CO_2H)^+$ or $Cl^-(C_2H_6O_2N)^+$, and $(NH_2CH_2CO_2)^-Na^+$ or $(C_2H_4O_2N)^-Na^+$, or $(HONH_3CH_2CO_2)^-Na^+$ or $(C_2H_6O_2N)^-Na^+$, omitting possible hydration of the ions.

The consideration of the transition point spoken of in connection with the different kinds of ionization is a more difficult one. It can best be treated from the side of the isoelectric points of the amphoteric substances.

The definitions of isoelectric point given by various workers have been contradictory at times. The most satisfactory definition appears to be that hydrogen ion concentration at which the properties such as electrical conductivity, viscosity, solubility, etc., when studied over extended ranges of acidity in moderately dilute solutions, show a point of inflexion.[19] Chemically this would be interpreted as stating that in more acid solutions the substance ionizes with the complex as part of the cation, in more alkaline solutions, as part of the anion. At the isoelectric point combination with added acid or base and accompanying ionization is a minimum, or the substance is in a maximum uncombined state. The method of cataphoresis in which the hydrogen ion concentration is determined at which the ampholyte does not migrate in solution or migrates in both directions under the influence

[20] Bredig, G., *Z. Elektrochem.*, 1899–1900, vi, 33.

[21] Walker, J., *Proc. Roy. Soc. London, Series B*, 1904, lxxiii, 155; 1905, lxxiv, 271; *Z. physik. Chem.*, 1904, xlix, 82; 1905, li, 706.

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of an electric current has been used to determine the isoelectric points of a number of proteins and other complex bodies.[10, 22]

The equation for calculating the isoelectric point of an amphoteric electrolyte

$$I = \sqrt{\frac{k_a}{k_b} k_w}$$

was deduced by Michaelis and Mostynski.[22] Their deduction does not appear to be altogether clear in so far as the assumptions involved are concerned. The following deduction brings out perhaps more satisfactorily these assumptions. It includes the view that the concentration of the unionized molecule in each case is given by the total number of molecules, not separate molecular species as indicated above.

$$k_a (HAOH) = (H^+)(AOH^-)$$

$$k_b (HAOH) = (OH^-)(HA^+)$$

$$\frac{k_a}{k_b} = \frac{(H^+)(AOH^-)}{(OH^-)(HA^+)}$$

$$= \frac{(H^+)^2}{k_w} \cdot \frac{(AOH^-)}{(HA^+)}$$

If

$$(AOH^-) = (HA^+)$$

$$(H^+) = \sqrt{\frac{k_a}{k_b} k_w} \qquad (1)$$

This derivation assumes the equalities of (HAOH) in the two ionization equations and of (AOH^-) and (HA^+). In the Michaelis and Mostynski original deduction, the assumptions were stated to be the equality of the complex anion and cation concentrations and a minimum total ionization of the ampholyte.

The following deduction involves the view of different molecular species ionizing as acid and as basic salts.

[22] Hardy, W. B., *J. Physiol.*, 1898, xxiv, 288. Cohn, Gross, and Johnson,[2] p. 149.

[22] Michaelis, L., and Mostynski, B., *Biochem. Z.*, 1910, xxiv, 79.

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$$k_a\,(\mathrm{HAOH}) = (\mathrm{H^+})\,(\mathrm{AOH^-})$$

$$k_b\,(\mathrm{HA_1OH}) = (\mathrm{OH^-})\,(\mathrm{HA_1^+})$$

$$\frac{k_a}{k_b} = \frac{(\mathrm{H^+})\,(\mathrm{AOH^-})\,(\mathrm{HA_1OH})}{(\mathrm{OH})\,(\mathrm{HA_1^+})\,(\mathrm{HAOH})}$$

$$= \frac{(\mathrm{H^+})^2\,(\mathrm{AOH^-})\,(\mathrm{HA_1OH})}{k_w\,(\mathrm{HA_1^+})\,(\mathrm{HAOH})}$$

$$(\mathrm{H^+}) = \sqrt{\underset{(a)}{\frac{k_a}{k_b}\,k_w} \cdot \underset{(b)}{\frac{(\mathrm{HA_1^+})}{(\mathrm{HA_1OH})}} \cdot \underset{(c)}{\frac{(\mathrm{HAOH})}{(\mathrm{AOH^-})}}} \qquad (2)$$

Equation (2) differs from equation (1) in containing the additional terms (*b*) and (*c*). For the two equations to give the same isoelectric point in any given case either of the following relations must hold (these are fundamentally the same with the terms arranged differently).

$$\frac{(\mathrm{HA^+})}{(\mathrm{AOH^-})} = \frac{(\mathrm{HA_1OH})}{(\mathrm{HAOH})} \qquad (3)$$

$$\frac{(\mathrm{HA_1^+})}{(\mathrm{HA_1OH}) + (\mathrm{HA_1^+})} = \frac{(\mathrm{AOH^-})}{(\mathrm{HAOH}) + (\mathrm{AOH^-})} \qquad (4)$$

From equation (4) it is evident that the degrees of ionization of the two molecular species are the same. This states nothing in regard to the concentrations of the ions or of the molecular species from which they are derived. Also, from equation (3), the ratio of the ampholyte ion concentrations is equal to the ratio of the corresponding unionized molecular species concentrations. That is to say, a greater ionic concentration of the ampholyte as acid species is accompanied by a greater relative concentration of the corresponding unionized molecular species. Obviously, also, equations (1) and (2) are identical if $(\mathrm{HA_1^+}) = (\mathrm{AOH^-})$ and $(\mathrm{HA_1OH}) = (\mathrm{HAOH})$, the assumptions under which equation (1) was deduced.

Equations (1) and (2) do not give the same isoelectric point, if the reciprocal of (*c*) is larger than (*b*), or the ionization as acid is greater than as base. The product of (*b*) and (*c*) will then be less than unity and the value obtained by means of equation (2) will be less than that given by equation (1). The reverse relation holds similarly.

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Because of insufficient data, the application of these relations is possible only in isolated cases. For simple substances, including amino-acids, etc., the isoelectric point as defined is probably identical with the hydrogen ion concentration of the pure substance dissolved in water. For more complex substances such as proteins, with a number of different acid- and base-combining groups, there will probably ordinarily be an overlapping of actions. The isoelectric point will then be the hydrogen ion concentration which involves a minimum combination with added acid or alkali, where the protein exists most nearly uncombined. The isoelectric point of a substance obviously shows the relative strengths of the substance acting as an acid and as a base.

Equation (1) requires that the isoelectric point of an ampholyte does not change with change in concentration.[24] Some results with glycine and asparagine show definite if small changes in the hydrogen ion concentrations of solutions of these substances on dilution.[25] The use of equation (2) may help to explain these variations.

The agreement between the isoelectric points calculated by means of equation (1) and those found experimentally is surprisingly close in many cases. For substances such as glycine, etc., where the values of the acid and basic dissociation constants are not far removed from each other and the isoelectric points in the neighborhood of the hydrogen ion concentration of the solvent, this is not unexpected. For a substance like aspartic acid, for which $k_a^{26} = 1.5 \times 10^{-4}$ and $k_b^{26} = 1.2 \times 10^{-12}$, the calculated isoelectric point according to equation (1) is very nearly $(H^+) = 10^{-3}$N. The value found by the indicator method was $(H^+) = 10^{-2.9}$N. This can only mean that there is some sort of compensation with terms (*b*) and (*c*) of equation (2) resulting in the calculated values differing to only minor extents from those given by equation (1). It must be remembered, however, that this compensation is not a necessary conclusion in every case as far as known at present, but that differences may be shown by the two equations.

[24] *Cf.* Tizard, H. T., *J. Chem. Soc.*, 1910, xcvii, 2490.

[25] Quoted by Clark,[2] p. 30, from results of S. P. L. Sörensen.

[26] Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546. Lundén, H., *Z. physik. Chem.*, 1906. liv, 532; *J. Biol. Chem.*, 1908, iv, 287.

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SUMMARY.

The titration curves of solutions of glycine, alanine, α-amino-butyric acid, leucine, glycyl-glycine, alanyl-glycine, alanyl-alanine, acetone, acetamide, urea, acetic acid, and aceturic acid were determined and some of the relations as dependent upon the chemical structures discussed.

The isoelectric points of some of the amphoteric electrolytes were found experimentally. The definition of isoelectric point, its theoretical significance, and method of calculation were considered in some detail.

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THE ISOELECTRIC POINT OF RED BLOOD CELLS AND ITS RELATION TO AGGLUTINATION.

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INTRODUCTION.

A number of investigations have been made into the electrical charges carried by bacteria and other cells in watery suspension. The movement observed in cataphoresis is affected by a number of factors, the analysis of which is so difficult that we have at the present time very little information as to the relation between agglutination and the electrical charge of the cells. Cellular suspensions behave in many ways like colloidal solutions, and it is generally understood now that colloidal protein solutions are amphoteric electrolytes. Their colloidal particles carry a negative charge in alkaline reactions and a positive charge in acid reactions; at a definite hydrogen ion concentration there exists no difference in electrical potential between the particles and the medium, so that the particles appear uncharged. This is known as the isoelectric point. At this point a number of physical properties such as solubility, viscosity, and conductivity pass through a minimum. Further, as shown by Loeb,[1] the isoelectric point is a turning point for the chemical change that determines the nature of the ionization. It is to this ionization that the electrical charge is due, so that when ionized as an acid and combined with the cation on the alkaline side of the isoelectric point the protein particle behaves as an anion and moves in the electric field to the anode; when ionized as a base and combined with anion, at reactions more acid than the isoelectric point, the protein becomes part of a complex

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[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

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cation and moves to the cathode. Chemically, therefore, the protein exists most nearly "pure," least combined with acid or alkali, at the isoelectric point. It is the purpose of the present paper to show the applicability of this conception of the physical and chemical significance of the isoelectric point to the agglutination of red cell suspensions.

The literature pertaining to this subject may be reviewed briefly. The movement of red blood cells in the electric field has been observed by Lillie,[2] by Girard-Mangin and Henri,[3] and by others since. Höber[4] found that the original negative charge became positive on saturation of the solution with CO_2 in the presence of very small amounts of electrolyte, but found that the charge was not reversed if large amounts of salt were present. This effect he referred to the acid character of the CO_2 introduced, and ascribed the reversal of charge to the permeability of the cell wall to anions at the increased hydrogen ion concentration. He noted that ferric and aluminium salts conferred a positive charge upon the cells in all concentrations of NaCl in the solution. Mines[5] found the red blood cells of *Scyllium* negatively charged in the serum. On the addition of strong cerium chloride solution to a suspension of cells the charge becomes uniformly positive and no agglutination occurs; with dilute cerium solution the cells promptly agglutinate. This Mines explains by assuming that in dilute cerium solution only certain of the cells are affected; these cells encounter others still negatively charged and mutual neutralization results, with precipitation of the neutralized cells.

Electric transport of bacteria has been carried out by many investigators. Sears and Jameson,[6] pupils of Zinsser, studied the migration of bacteria at varying degrees of acidity and found that addition of alkali increased their rate of movement while acid lessened it. The fact that protein solutions flocculate most readily near their isoelectric point has suggested that this may be the mechanism of bacterial agglutination, but Beniasch[7] in studying acid agglutination of bacteria

[2] Lillie, R. S., *Am. J. Physiol.*, 1903, viii, 273.
[3] Girard-Mangin, and Henri, V., *Compt. rend. Soc. Biol.*, 1904, lvi, 866.
[4] Höber, R., *Arch. ges. Physiol.*, 1904, ci, 607; 1904, cii, 196.
[5] Mines, G. R., *Kolloid Chem.*, 1911–12, iii, 191, 236.
[6] Sears, H. J.,and Jameson, E., Cataphoresis of Bacteria, San Francisco, 1912.
[7] Beniasch, M., *Z. Immunitätsforsch., Orig.*, 1911, xii, 268.

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found that alterations in the hydrogen ion concentration produced no change in the electrical properties of the bacteria; their original negative charge was not neutralized at the optimal point for agglutination and was not reversed by a more acid reaction. Arkwright[8] found, however, two optimal zones for acid agglutination of typhoid bacilli; in the more acid zone (pH 3.0) agglutination of the bacterial bodies is associated with absence of electrical charge. In the less acid zone (pH 4.7 to 3.5) a soluble bacterial protein precipitates and no change in charge is noted.

As to the influence of specific sensitization very little is known. Neisser and Friedemann[9] observed that agglutinin bacteria flocculate in the electric field as a direct result apparently of the electric flow. Michaelis and Davidsohn[10] conclude that specific typhoid agglutination and precipitation are independent of the H concentration, although from their tables for agglutination it appears that there is an optimum for this phenomenon lying between pH 4.6 and 3.7 very close to that of the native bacteria, which is at pH 4.4.

EXPERIMENTAL.

The isoelectric point was determined in the present work by the method of cataphoresis, using a model of U-tube evolved after experiments with many types of apparatus. It is illustrated in Fig. 1. The non-polarizable electrodes consist of zinc rods inserted through perforated rubber stoppers into the long ends of large stop-cock tubes. The lower portion of these tubes and the cock itself are filled with 15 per cent zinc sulfate solution. The upper part of these tubes contains a buffer solution of acetic acid acetate or phosphate mixture. This solution is made up to 10 per cent of saccharose, in order to have a specific gravity less than that of the zinc sulfate and greater than that of isotonic (M/4) saccharose solution alone, to minimize diffusion when the different fluids are in contact. The U-tube itself has an inside diameter of 6 mm. and a total length of 30 cm. It is made in two equal portions, so that after filling the side arms including the

[8] Arkwright, J. A., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 396.

[9] Neisser, M., and Friedemann, U., *Münch. Med. Woch.*, 1904, li, 465, 827.

[10] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

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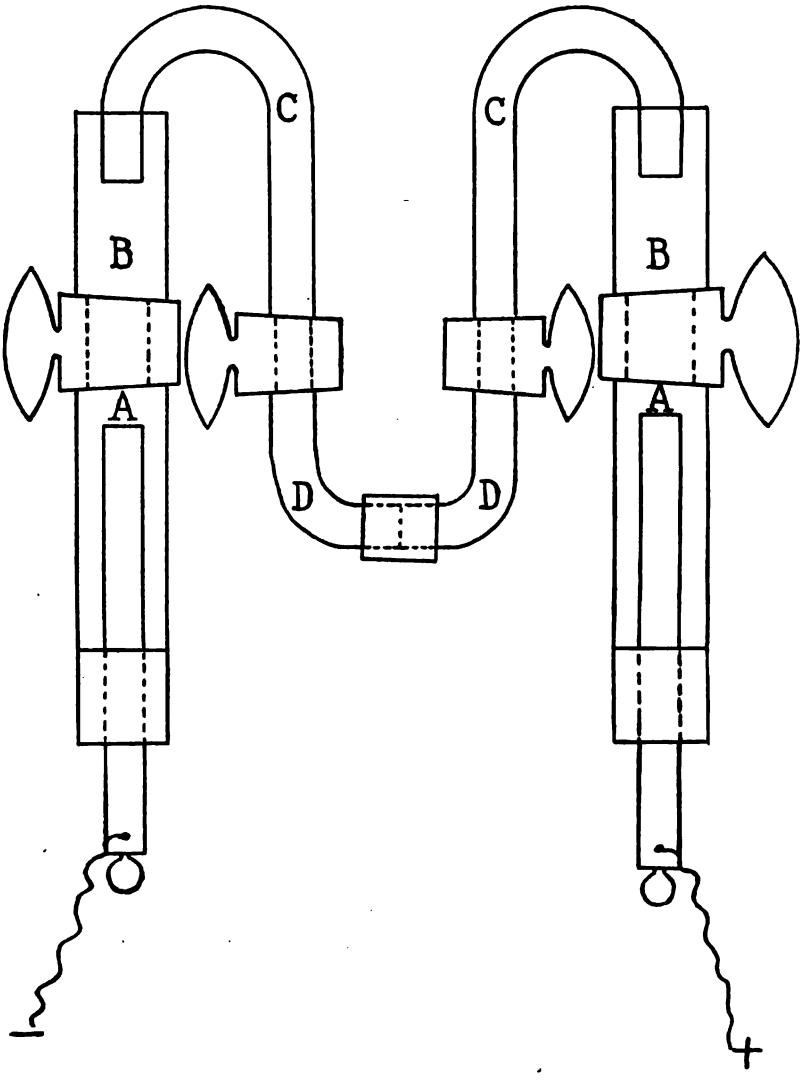

FIG. 1. Apparatus for cataphoresis of red blood cells. A, $ZnSO_4$; B, buffer; C, saccharose; D, cell suspension.

small stop-cocks with solution, the two halves of the middle portion can be filled separately with the test suspension and then pressed together, the short length of rubber tubing being left attached to one

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of the halves. The ends of the side arms are then hooked into the electrode tubes containing buffer solution, and the cocks carefully opened. The fluid in the middle portion is brought to the same level on each side by adding a small amount, one or two drops, of buffer solution to the buffer chamber on one side or the other. With this type of apparatus the fall in electrical potential across the U-tube is relatively great, and the change in hydrogen ion concentration in the test fluid during the passage of current is reduced to a minimum. The determinations were made at 19 ± 1°C. without a thermostat in a room of even temperature. The current used was the Edison direct street current of 220 volts, giving a drop of almost exactly 7 volts per cm. in the U-tube.

Fresh defibrinated sheep blood was washed with two or more changes of saline solution and then freed from electrolyte by washing with four changes of ten volumes each of isotonic saccharose solution (M/4), and finally made up to 10 per cent concentration of cells in saccharose. It was found that the reaction of the saccharose solution should not be more alkaline than pH 6.5 as at more alkaline reactions the viscidity of the sedimented cell mass is so great that it is reemulsified only with difficulty and with the development of considerable hemolysis.

To determine the direction and rate of movement of red cells at varying hydrogen ion concentrations, a titration was first carried out to determine the H concentration resulting from the addition of graded amounts of HCl to the cell suspension at hand. 5 cc. of cells were introduced into twelve or more tubes, in two series. To the first series of tubes were then added 5 cc. of saccharose solution and increasing amounts of M/10 or M/100 HCl, the latter dilution made up in saccharose. Between manipulations the tubes and the stock flask were kept tightly stoppered to exclude atmospheric CO_2 as far as possible. After gentle agitation the tubes were allowed to stand at room temperature for 10 to 15 minutes, then centrifugated and the supernatant fluid was drawn off. Of this, one portion of 5 cc. with indicator added was compared with a standard series for the colorimetric determination of hydrogen ion concentration, the remaining portion serving as a color screen for the standard tube. The value thus obtained represents the H concentration with which the cells were in equilibrium.

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The test emulsion for cataphoresis was prepared by adding the proper amount of acid to secure the desired H concentration to 5 cc. of saccharose solution; this was then poured gently into one of the second series of tubes of cells, and the mixture pipetted immediately into the U-tube. In this way it was possible even at reactions near the isoelectric point to observe the movement of cells before agglutination was complete and in many cases before macroscopic agglutination had appeared. For each determination 10 cc. of buffer solution and 10 cc. of saccharose solution, of the H concentration of the tube of cells to be examined, were prepared. If the apparatus was carefully manipulated the boundary of contact between the cells and the fluid in the side arms remained sharp, and the distance of displacement of the boundary during passage of current could be measured quite accurately with a pair of dividers. When agglutination appeared, a more dilute suspension remained, the movement of which could be determined likewise.

In addition to the determinations made in the absence, as far as possible, of electrolyte, measurements were made of the migration of cells in the presence of sodium chloride, sodium acetate, and sodium phosphate, using a dilution of 1 part of isotonic solution of these salts to 4 parts of saccharose solution. The acetate and phosphate were used in the form of buffer mixtures.

Sensitized cells were prepared by adding to a 10 per cent suspension of cells in saccharose approximately 50 hemolytic units of a high titer immune rabbit or hare serum. The reaction of the mixture was about pH 6.0. After incubation for 2 hours at 37°C. the cells were either used at once or were placed in the refrigerator over night. Before use the cells were sedimented twice and again made up to 10 per cent concentration. The amount of electrolyte in the immune serum was insufficient to cause agglutination. Kosakai[11] has shown that sensitizer can be dissociated from red cells by extraction in salt-free solutions of various sugars; we have been able to confirm this, but the dissociation is not complete and the cells used in our determinations were still combined with sensitizer, as shown by prompt hemolysis on the addition of complement and by agglutination when added to saline

[11] Kosakai, M., *J. Immunol.*, 1918, iii, 109.

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solution of the usual strength. The titrations for H concentrations of the sensitized cells were carried out according to the method already described.

The determinations for normal and sensitized cells have been recorded in the form of curves (Figs. 2 and 3) in which the millimeters of movement during 10 minutes time are plotted as ordinates against the pH values as abscissæ. From these curves it appears that the

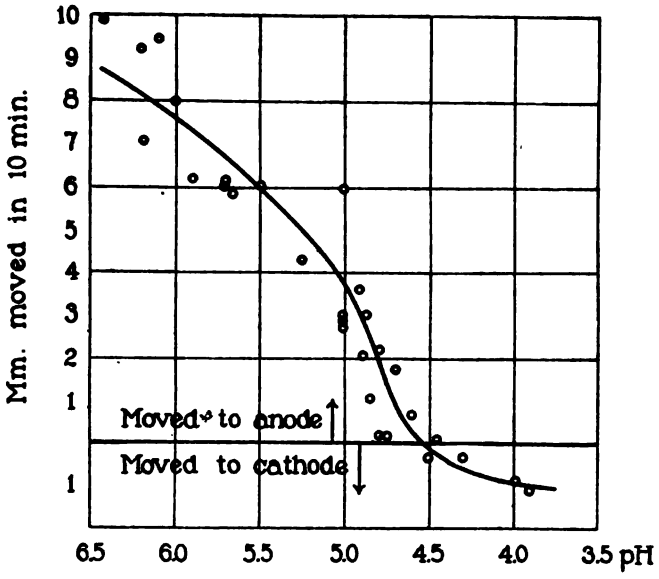

FIG. 2. Curve showing the movement in mm. during 10 minutes of normal red blood cells at varying hydrogen ion concentrations. The points above the curve between pH 4.0 and 5.0 represent determinations on different specimens of blood from those recorded in the points below the curve, so that the difference may represent actual variations in individual specimens.

direction and rate of movement in the electric field of both normal and sensitized red blood cells is a function of the hydrogen ion concentration. At concentrations less than pH 4.6 the charge carried is negative and increases in amount with the alkalinity; pH 4.6 represents the isoelectric point; at concentrations greater than pH 4.6 the charge carried is positive and increases in amount with the acidity.

A comparison of the two curves shows that on the alkaline side of the isoelectric point the charge of normal cells is greater and increases

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more rapidly with increasing alkalinity than the charge of sensitized cells. The slower movement cannot be explained by the formation of agglutinated masses, since at the upper end of the curve at least no agglutination appeared even after 12 hours standing in the U-tube. On the acid side of the isoelectric point the measurements are not numerous and probably not accurate enough for a comparison.

The optimum H concentration for agglutination was determined by titrations exactly like those described above. Varying amounts of cells were used; the sharpest results were obtained by adding 1 or 2

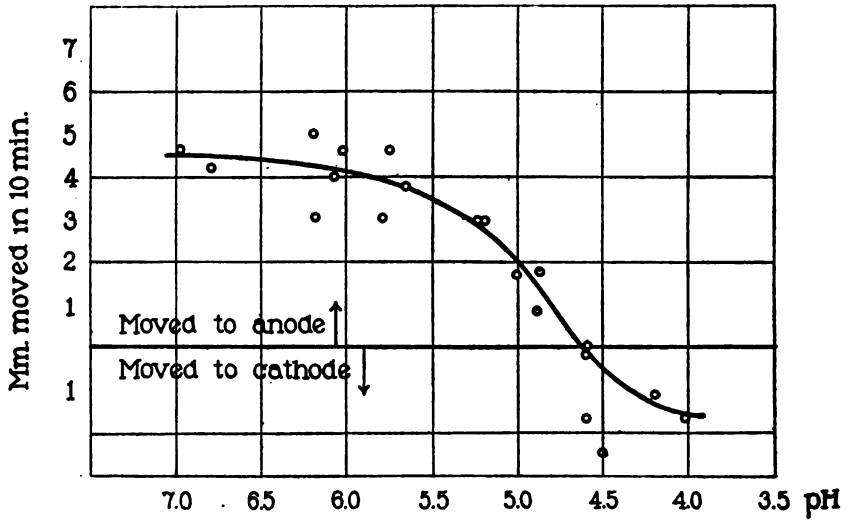

FIG. 3. Curve showing the movement in mm. during 10 minutes of sensitized red blood cells.

cc. of 10 per cent cells to 10 cc. of saccharose solution containing increasing amounts of HCl. At the optimum concentration agglutination is almost instantaneous; at greater and less acidities agglutination appears more slowly, but was practically completed within 30 minutes. The agglutination arranges itself asymmetrically on either side of the optimal point; with normal cells it never was observed at reactions more alkaline than pH 5.6, and seldom beyond pH 5.1. This variation is probably connected with variations in the amount of electrolyte present, and will be considered later. On the acid side

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of the optimal point, however, agglutination appeared at all concentrations. As a result of over thirty series of determinations the optimum for agglutination of normal red cells may be given as pH 4.75. In the great majority of experiments the optimum corresponded quite sharply with this figure.

With sensitized cells, a greater variation was noted in individual experiments, due possibly to varying degrees of sensitization, but when the cells were still heavily sensitized after the final washing, the optimum for agglutination occurred regularly near pH 5.3. The optimum for precipitation of serum globulin, in which fraction of the serum the immune bodies are carried, is given as pH 5.2 by Rona and Michaelis,[12] and the isoelectric point of typhoid immune body[10] as pH 5.4 so that the flocculation of sensitized cells seems to be connected with that of the specific immune serum.

The Chemical Significance of the Isoelectric Point.

From the data given by the titrations above described, and carried out for the determination either of the isoelectric point or the optimum for agglutination, one may construct a curve giving the amounts of HCl added, as ordinates, on the pH values attained, as abscissæ. A similar curve may be drawn for the addition of acid to 10 cc. of saccharose solution alone. From these two curves a third may be drawn (Fig. 4) with pH values again as abscissæ, and as ordinates the differences between the ordinates of the first two curves, or the amounts of HCl required to bring a suspension of cells to a given H concentration in excess of those necessary in the case of the saccharose solution alone. There is evident a sharp inflection in this curve at pH 4.7. This point is so close to the value found for the isoelectric point that we may speak of it as the same. The protein of the cells exists then on the acid side of the isoelectric point as a different chemical substance from that occurring on the alkaline side. Since a true "puffer" action is manifest, it appears that the protein combines with hydrogen ion. An adsorption of hydrogen ion can hardly be thought of here as anything but a chemical combination.

[12] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

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The observation by Joos[13] that the salt necessary for the agglutination of sensitized bacteria combined chemically with the bacteria, since it was not to be detected in the supernatant fluid after agglutination, although not confirmed by later investigators, led me to investigate the removal of salt after acid agglutination. With cells washed as free as possible from electrolyte, the amount of chloride present was very small and consisted chiefly in that added as HCl. A test for chlorine ion was made with $AgNO_3$ on the supernatant fluid from the cells, and it was found that at pH 4.8 no chloride was present, and

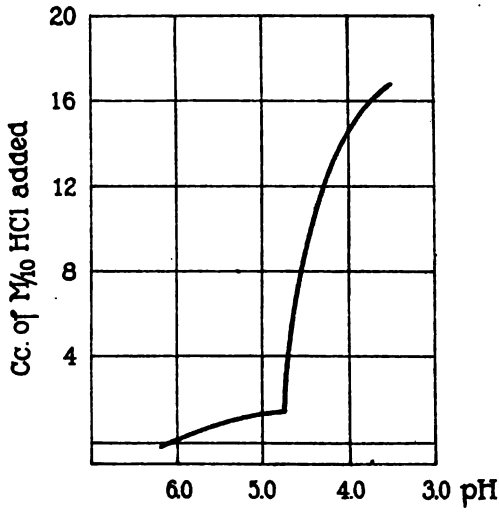

FIG. 4. Curve showing the combination of red blood cells with hydrogen ion. 5 cc. of 10 per cent normal cells. The same curve is given with sensitized cells.

only the slightest trace in the tubes to which considerable amounts of HCl had been added, while on the alkaline side of pH 4.8 a distinct turbidity appeared.

This experiment was repeated many times, always with the same result. The conditions were determined more accurately by volumetric analysis of the fluid from tubes of cells to which small amounts of M/10 NaCl were added along with M/10 HCl, so that each tube contained exactly the same amount of chlorine ion. The results are

[13] Joos, A., *Z. Hyg.*, 1901, xxxvi, 422.

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given in the form of curves, Fig. 5 being that for normal cells and Fig. 6 that for sensitized cells. The curves give as ordinates the gram-ions $\times 10^{-5}$ of Cl lost by the supernatant fluid, or gained by the cells. It will be seen that the chlorine ion combines with both normal and sensitized cells in much larger amount on the acid side of pH 4.7 than

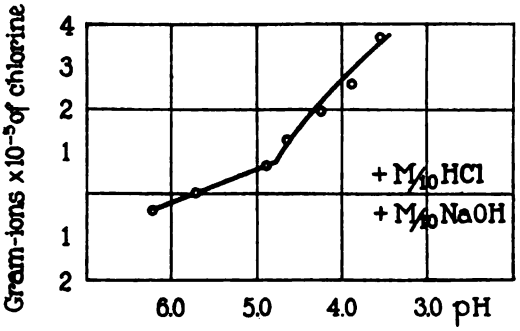

FIG. 5. Curve showing the combination of normal red blood cells with chlorine ion. 5 cc. of 10 per cent cells.

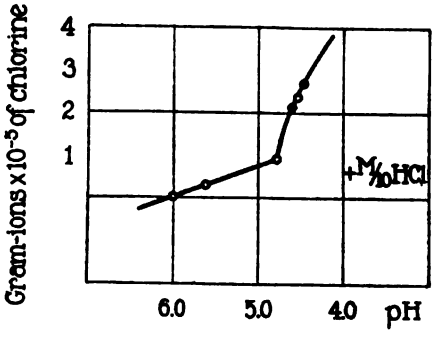

FIG. 6. Curve showing the combination of sensitized cells with chlorine ion. 5 cc. of 13 per cent cells.

on the alkaline side. On adding NaOH, Cl is actually given up by the cells between pH 5.7 and 6.2. This effect cannot be due merely to an increase in permeability of the cells, permitting the escape of chlorine ion, for in that case the amount of chlorine ion actually found should have been greatest at pH 3.5, where a trace of hemolysis appeared.

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A similar determination was made on the combination with a cation at different pH values. Cells were suspended in isotonic $BaCl_2$, then washed, and again suspended in saccharose solution which had been brought to pH 6.0 with $Ba(OH)_2$ instead of NaOH. The amount of Ba present was therefore small, and could only be estimated from the turbidity of the fluid on adding Na_2SO_4. Many of the experiments showed no significant differences; in several, however, it was found that the amount of Ba in the supernatant fluid increased sharply at pH 4.7 and showed a further increase to pH 4.0. This must mean that the Ba was liberated from combination with the cells on the acid side of the isoelectric point.

Exactly the same chemical behavior has been found by Loeb[1] for gelatin, which combines with cation only on the alkaline side of its isoelectric point and with anion only on the acid side. This Loeb has shown by a series of volumetric analyses for Ag, Br, and CNS ions of the gelatin-ion compound. This work was unfamiliar to the present author at the time the observations detailed above were carried out. It is significant of the general importance of this relation that the same chemical behavior is manifested both by solutions of gelatin and by living cells. The old observation of Hamburger[14] that under the influence of HCl, H_2SO_4, or CO_2 red cells take up chlorine from the serum, while on the addition of alkali they give chlorine up to the serum, is thus to be interpreted, as suggested by Loeb, by the difference in chemical combining power on either side of the isoelectric point.

The conclusion appears justified by these facts that the character of the charge carried by a red cell depends upon the nature of its chemical combination; and that the amount of charge depends upon the amount of protein in ionic combination. This conclusion was facilitated by the observations of Loeb, already referred to.

The curve for velocities of normal cells (Fig. 2) on the alkaline side of the isoelectric point closely parallels the curve given by Loeb for swelling and conductivity of Na gelatin. Korányi and Bence[15] have shown that between saturation of defibrinated blood with CO_2 on the

[14] Hamburger, H. T., Osmotischer Druck und Ionenlehre, Wiesbaden, 1902, i.
[15] Korányi, A., and Bence, J., *Arch. ges. Physiol.*, 1905, cx, 513.

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one hand and removal of the CO_2 by a stream of oxygen, on the other, the values for viscosity of the blood and refractive index of the serum pass through the minima. From the data already given, it appears that we must interpret these minima as corresponding with the isoelectric points of the serum and the cells, although in the lack of definite values for the reaction at which these minima occurred, this conclusion must be considered probable rather than proved. Indeed this conclusion was foreshadowed by Korányi and Bence in their interpretation of Höber's observations[4] on the migration of red cells.

DISCUSSION.

The effect of the addition of sodium chloride, sodium phosphate, or sodium acetate upon the velocity of cells, on the alkaline side of the isoelectric point at least, is a minor one. As compared with the values in the absence of electrolyte the velocities are somewhat greater in the presence of NaCl, and somewhat less in the presence of the other salts. These variations are in all probability due to alterations in the viscosity of the medium, which was not corrected for in the measurements given in the curves. This close correspondence of values is what we should expect if the cells exist at these reactions only as sodium proteinate, in these experiments.

When normal cells are washed in four or more changes of saccharose solution, a very slight absorption of CO_2 from the atmosphere is sufficient to raise the H concentration to pH 4.75, at which the cells agglutinate almost instantaneously. In this condition they exist most nearly in the pure state, uncombined with acid or base.

The addition of NaCl on the other hand lessens their susceptibility to change in reaction. When this salt or $CaCl_2$ is added the pH reached is less acid for a given amount of HCl than if the cells are suspended in saccharose solution alone. It is this decrease in H concentration in the presence of salt, without doubt, which carried the reaction in Höber's experiments from the acid to the alkaline side of the isoelectric point and thereby prevented the saturation of the solution with CO_2 from bringing about a positive charge on the cells. The pH value for the isoelectric point is not altered, but it is attained only on the addition of larger amounts of HCl. The curve for pH

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values shows the same inflection at pH 4.7 as Fig. 4, and is therefore omitted.

This effect is very difficult to analyze. It appears to be a chemical one, and may involve the formation of a small amount of protein chloride on the alkaline side of the isoelectric point, due to the excess of chlorine ion, but since the effect is noted on both sides of the isoelectric point, any explanation of it in the light of the facts presented here would be largely speculative.

The agglutinability of sensitized cells in the presence of an electrolyte as compared with the stability of normal cells is not an absolute difference, even when the electrolyte is NaCl. Specific agglutination is known to occur over a wide range of pH values, as shown by Krumwiede and Pratt[16] among others; that it is not independent of the hydrogen ion concentration, however, is indicated as shown above by the existence of an optimum, at which it is practically independent of the presence of neutral salt. On either side of this range as common experience shows it is accelerated by the presence of neutral salt. Similarly normal cells which may not agglutinate in salt-free media at pH 4.9 have been found in our titrations to agglutinate readily at pH 5.5 in the presence of NaCl. At less acidities, while flocculation may not occur, we have found the speed of settling to be affected in a similar way by the hydrogen ion concentration.

It is apparent then when we compare the influence of the hydrogen ion concentration upon the electrical charge and chemical behavior of red cells with the influence upon agglutination that we have to deal not with one phenomenon, but with two, which are, however, closely related.

CONCLUSIONS.

1. The movement of normal and sensitized red blood cells in the electric field is a function of the hydrogen ion concentration. The isoelectric point, at which no movement occurs, corresponds with pH 4.6.

2. On the alkaline side of the isoelectric point the charge carried is negative and increases with the alkalinity. On the acid side the charge is positive and increases with the acidity.

[16] Krumwiede, C., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1912, xvi, 517.

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3. On the alkaline side at least the charge carried by sensitized cells is smaller and increases less rapidly with the alkalinity than the charge of normal cells.

4. Both normal and sensitized cells combine chemically with inorganic ions, and the isoelectric point is a turning point for this chemical behavior. On the acid side the cells combine with the hydrogen and chlorine ions, and in much larger amount than on the alkaline side; on the alkaline side the cells combine with a cation (Ba), and in larger amount than on the acid side. This behavior corresponds with that found by Loeb for gelatin.

5. The optimum for agglutination of normal cells is at pH 4.75, so that at this point the cells exist most nearly pure, or least combined with anion and cation.

6. The optimum for agglutination of sensitized cells is at pH 5.3. This point is probably connected with the optimum for flocculation of the immune serum body.

The author wishes to express his indebtedness to Dr. K. George Falk, of the Harriman Research Laboratory, the Roosevelt Hospital, New York, for criticism and suggestions.

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THE PRODUCTION OF VOLATILE FATTY ACIDS BY BACTERIA OF THE DYSENTERY GROUP.

By HARPER F. ZOLLER and W. MANSFIELD CLARK.

(From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture, Washington.)

(Received for publication, October 11, 1920.)

INTRODUCTION.

In the course of an investigation of bacteria of the dysentery group we had occasion to determine the volatile fatty acids produced by these bacteria under different conditions. We believe the results to have considerable significance but their interpretation had best wait upon the development of more fundamental knowledge of anaerobiosis than we now possess. We therefore desire to place the following data on record without comment.

Organisms Studied—The cultures were secured from several investigators and institutions and represented the usual types together with some atypical organisms. A laboratory fire destroyed the history of each culture, but they have been separated into Shiga and non-Shiga types by mannite fermentation tests through the cooperation of Dr. J. M. Sherman of this laboratory. Fermentation data were at hand which would permit the differentiation of the non-Shiga cultures into Flexner, Strong, and other strains; but this was not done, since the results of the volatile acid work are in such close agreement that, as conducted, they have no important bearing upon the classification of the dysentery bacteria. The results from one each of typhoid (Ty Round) and paratyphoid (P Ty 16) cultures are included because of the relation existing between the dysentery and these groups of organisms. Following the laboratory number will be found the strains studied: D_1, Flexner; D_2, Shiga; D_3, Shiga; D_4, Flexner; D_5, Shiga; D_6, non-Shiga; D_8, non-Shiga; D_{10}, non-Shiga; D_{11}, non-Shiga; D_{13}, non-Shiga; D_{17}, non-Shiga; D_{22}, non-Shiga; D_{24}, Shiga; D_{33}, non-Shiga; D_{37}, Shiga.

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Methods.

Media.—The medium for the growth of the organisms consisted of 1.0 per cent of "Difco" peptone, and 0.5 per cent of $Na_2HPO_4 \cdot 2H_2O$, with or without 1.0 per cent of Merck's "highest purity" dextrose. The reaction of this medium after sterilization always lay between pH 7.1 and 7.2. The hydrogen ion concentrations of the media and cultures were determined colorimetrically.

Cultures.—The organisms were inoculated into sterile 10 cc. portions of the media and incubated at 37°C. for 18 hours. These virile cultures were then poured into 990 cc. of the sterile media contained in 2 liter Florence flasks and incubated at 37°C. for the period of time indicated. The pouring was conducted under conditions which reduced the chance for contamination by this method. No control plates were run to determine this factor, since it was held that the bulky inoculation was sufficient to take care of any slight contamination should such occur. The regularity in the content of volatile fatty acids leads us to believe that there was no contamination. It was found that the maximum production of total volatile acids in sugar medium under these conditions occurred shortly before the first 12 hour period and the total quantity remained quite stationary during the following 60 hours (Fig. 1). Accordingly, the cultures were allowed to become 48 hours old merely as an arbitrary period. The cultures in non-sugar media were allowed a longer period before examining for volatile acids, because volatile bases were investigated in the same cultures. It is barely possible that the values for the volatile acids obtained in the case of these cultures grown for a longer period of time are somewhat too low because of the chance of volatile acid utilization.

Anaerobic Cultures.—"Anaerobic" conditions were obtained by evacuation of the inoculated flasks with a mercury-vapor pump (a single Kraus modeled pump) in series with a motor-driven Geryke vacuum pump. Gum stoppers, bearing a thick-walled tube drawn out to a narrow neck to permit sealing with a fine tipped flame, were cemented while hot with a heavy rubber-rosin cement into the necks of the flasks. These stoppers had been previously sterilized, and the glass tubes contained a plug of cotton to prevent contamination of the

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flasks while evacuating. After pumping out the bulk of the air with the Geryke pump till the Plücker tube discharge showed a declining spark, the mercury-vapor pump was started and it was allowed to pump for 20 minutes more or until the Plücker tube discharge showed a continued decline in brilliancy. The flasks were sealed off while the pumps were in operation.

Separation of the Volatile Acids from the Cultures.—10 per cent sulfuric acid was added to the cultures until a drop of thymol blue showed a distinct red; this represented a pH of 1.0 or less. Steam formed by boiling distilled water in the presence of barium hydroxide was led into the flasks holding the cultures. The flasks in turn were connected to vertical condensers through which ice-cooled water circulated. The condensers were arranged vertically to facilitate the delivery of the condensed fatty acids. A small piece of paraffin was placed in the cultures to overcome frothing during distillation. The volume of liquid in the culture flasks was held constant by means of a screened flame. Fractions of 500 cc. each were caught and titrated with 0.1 N NaOH to phenolphthalein. The distillation was continued until the last fraction required no more than 2 cc. of 0.1 N NaOH. Usually ten to fourteen fractions were collected. These were evaporated *in toto* and made up to a definite volume.

Distillation was conducted upon a like volume of sterile medium to determine the factor for carbon dioxide, etc.

Quantitative Analysis of the Volatile Fatty Acids.—The modified Duclaux method published by Gillespie and Walters[1] was followed in the main in the determinations. It was found advisable to determine new constants for formic, acetic, propionic, and normal butyric acids with the distillation apparatus employed. The still was of Jena glass throughout and contained a small, closed end, inverted tube in the solution to insure against excessive spattering and to produce a steady flow of vapor. The constants found did not depart radically from those obtained by Gillespie and Walters. The still flask was heated on the sides and bottom with electrically heated coils.

An aliquot of the total volatile acid concentrate was employed which contained from 60 to 80 cc. of 0.1 N volatile acids.

[1] Gillespie, L. J., and Walters, E. H., *J. Am. Chem. Soc.*, 1917, xxxix, 2027.

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The mode of treating the results depended somewhat upon the number of volatile acids found to be present in the cultures under examination. For only two acids the algebraic method[1] (page 2036) was used, although the graphical method (page 2040) was found to be somewhat more rapid. When three acids were found to be present the graphical method mentioned by Gillespie and Walters (page 2054) was followed. This latter method involved much less calculation than the algebraic and was equally accurate.

As a check on the modified Duclaux method, the formic acid was determined in several cultures by the mercuric chloride method of Franzen and Egger.[2] The two methods gave values for this acid harmonizing within 2 per cent of the whole. It is assumed that the values found for the other acids are as accurate.

In the cultures containing butyric acid its presence was further established by preparing the quinine salt as outlined by Phelps and Palmer[3] and determining its melting point. The distillation values accurately establish the identities of the acids, provided the manipulations are carefully conducted and the constants are determined with purified acids.

Sugar determinations were conducted upon the media and several cultures to obtain data regarding the utilization of the sugar and the amount of acid formed therefrom. From 30 to 40 per cent of the sugar was utilized by the bacteria in cultures 2 and 14 days old. It was at first feared that, because the total quantity of volatile acids produced by each organism in the same length of time reached about the same value, not enough glucose was present. It is evident, however, if we view Fig. 1 in the light of this sugar utilization, that the organisms growing in sugar media reached their limiting zones of volatile fatty acid production in the presence of sugar. This may have an important bearing on the growth curve of bacteria. This large yield of formic acid by these bacteria from glucose suggests a possible commercial problem.

Table I contains a summary of the determinations under the four sets of conditions. Fig. 1 pictures the results of the progressive series

[2] Franzen, H., and Egger, F., *J. prakt. Chem.*, 1911, lxxxiii, 323.

[3] Phelps, I. K., and Palmer, H. E., *J. Biol. Chem.*, 1917, xxix, 199.

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studies and is included to furnish justification for choosing the 48 hour cultures for the main study.

TABLE I.

Volatile Fatty Acid Production of Dysentery Bacteria Under Different Conditions of Growth.

Organism.	Age of culture.	pH of culture.	Sugar present.	State.	Acid produced.			
					Formic. n/10	Acetic.	Propionic.	Butyric.
	days		*per cent*		*cc.*	*cc.*	*cc.*	*cc.*
D_1	2	4.9	1	Aerobic.	128.7	88.2	None.	None.
D_2	2	5.0	1	"	122.3	82.9	"	"
D_3	2	5.0	1	"	132.9	88.5	"	"
D_4	2	5.2	1	"	172.2	83.8	"	"
D_5	2	5.0	1	"	121.5	92.5	"	"
D_6	2	4.8	1	"	127.2	78.5	"	"
D_8	2	4.9	1	"	149.3	92.3	"	"
D_{10}	2	5.0	1	"	143.5	89.5	"	"
D_{11}	2	4.8	1	"	145.0	102.1	"	"
D_{12}	2	4.9	1	"	124.3	76.5	"	"
D_{17}	2	5.2	1	"	145.2	66.0	"	"
D_{23}	2	5.4	1	"	86.5	60.8	"	"
D_{24}	2	5.2	1	"	112.3	83.0	"	"
D_{33}	2	5.2	1	"	169.5	88.0	"	"
D_{37}	2	4.8	1	"	118.5	73.3	"	"
P Ty 16	2	5.1	1	"	77.3	80.2	"	"
Ty Round	2	5.1	1	"	150.5	78.6	"	"
D_1	10	7.5	None.	"	None.	33.2	12.8	"
D_2	10	7.6	"	"	"	35.2	13.3	"
D_4	10	7.5	"	"	"	27.9	11.1	"
D_{37}	12	7.4	"	"	"	31.5	11.8	"
D_1	12	6.2	"	Anaerobic.	31.2	27.1	None.	5.5
D_2	12	6.4	"	"	15.3	47.4	"	7.1
D_4	12	6.8	"	"	31.2	32.2	"	7.4
D_{37}	12	6.8	"	"	Determination lost.			
D_1	12	5.2	1	"	90.1	77.0	None.	None.
D_2*	14	5.2	1	"	87.5	56.5	"	"
D_4	12	5.5	1	"	116.4	78.2	"	"
D_{37}	12	5.4	1	"	94.8	68.5	"	"

* Growth less heavy to the eye.

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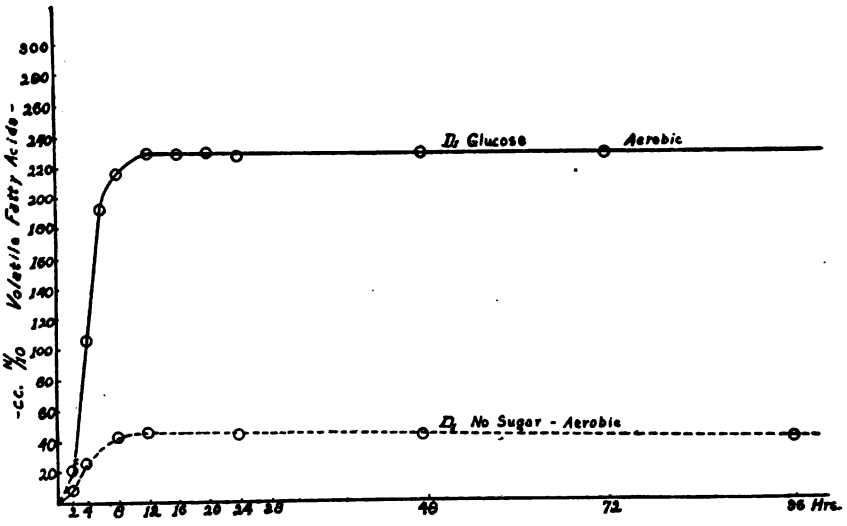

FIG. 1. Volatile fatty acid production curves of D_1 grown in 1,000 cc. of medium, and under the conditions indicated.

CONCLUSIONS.

These studies show:

1. A close agreement exists among all the organisms studied in the total quantity of volatile fatty acids produced and in the ratio of formic to acetic, under aerobic conditions, and in the presence of 1 per cent of glucose.

2. When grown upon peptone alone, with free access of air to the cultures, volatile fatty acids are produced in appreciable quantities, although the reaction of the solution has gone more alkaline as shown by colorimetric pH tests. Formic acid is not found, but in its place we obtain propionic acid.

3. Upon exhaustion of air from the non-sugar medium the bacteria again produce formic acid, and in addition some butyric. This is true for both Shiga and non-Shiga cultures. The reaction is distinctly more acid.

4. The presence of glucose in the medium from which the air has been pumped furnishes a condition which provokes about the same type and degree of fermentation that operates in the glucose medium bathed in air at atmospheric pressure.

5. The enormous quantity of formic acid produced by these bacteria may play a significant part in the digestive disturbances and toxic symptoms accompanying their infection of the human intestinal tract.

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THE RESPONSE OF POPILLIA JAPONICA TO LIGHT AND THE WEBER-FECHNER LAW.

By A. R. MOORE and W. H. COLE.*

(From the Riverton Entomological Laboratory, Riverton, N. J.)

(Received for publication, October 26, 1920.)

The Japanese beetle, *Popillia japonica*, is a recent importation from Japan and is found in a limited area in central New Jersey.

During July and the first half of August the beetles may be seen collected in great numbers at the tops of trees, bushes, and weeds. It seems to be immaterial whether the plant is a linden tree 50 feet high or a smartweed plant a foot high—the beetles always occupy the uppermost foliage. This is due to the fact that the direction of movement in these beetles is the additive resultant of two tropistic responses; namely, positive phototropism and negative geotropism. These tropisms determine the head-tail orientation of the body.

Both in the field and in the laboratory a certain degree of heat and of light is necessary for the active movements of *Popillia*. Thus, below 23°C. the beetles are generally inactive; 38–39°C. is optimum for their activity, while above 40°C. injurious effects are apparent. At 45°C. activity ceases quickly and permanently. Under the ruby light and in the dark most of the beetles become quiet and show no response to gravity. Occasional individuals which do move show a retarded response. All are roused to activity by illumination from any direction, while a great increase in the strength of the light causes marked acceleration of movement and flying.

In addition to this kinetic action of light there is also a directive effect, which may be demonstrated by illuminating the beetles first from above and then from below and timing their response in each case. Without exception they ascend the wire gauze of their cage more rapidly in the first instance, although in the latter case the

* The apparatus for this work was furnished by Rutgers College, New Brunswick, N. J.

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animals eventually reach the top, thus proving the geotropic response to be the major factor in orientation. Table I shows the retarding effect of illumination from below. Incidentally these figures well illustrate the constancy with which any given lot of animals responds. It is apparent from this result that while light exercises a directive action, the directive effect of gravity is greater than that of light of the strongest intensity used.

Since the geotropic response is shown by the beetles only when illuminated, it therefore follows that their movement in a lighted field is the result of three factors; *viz.*, negative geotropism, photo-

TABLE I.

*Light Intensity 3,276 Candle Meters.**

Light above.	Light below.
Reaction time.	Reaction time.
sec.	*sec.*
10	15
11	16
9	17
11	17
11	15
Mean.....10.4	16

* With the light above, gravity and light act together to produce a response in 10.4 seconds. When the light is below, the directive action of the light acts against the effect of gravity and thus delays the response to 16 seconds.

kinesis, and positive phototropism. The first is constant, the other two factors are functions of the intensity of the illumination. In the case in which the light is from above, it follows that changes in the rate of movement of the beetles depend on variations in the degree of photokinesis and of phototropism. These factors in turn depend upon the intensity of the illumination. Hence we may express the relation of the rate of response to the intensity of the illumination by saying that the rate of response is a function of the light intensity.

In the present study, the determinations of reaction time were made as follows. A cage of wire gauze 12.5 cm. high was constructed, having a movable top of the same material. During a series of

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experiments this cage containing twelve female beetles was kept in a thermostat made of glass at a temperature of approximately 38.5°C. with extreme variations for short intervals on two or three occasions during the season to 37.5 and 39.5°C. Five intensities of illumination were secured by the use of Mazda glowers of different powers. During the experiment the glower was held by a clamp 25 cm. from the top of the cage. The latter was protected from the heat of the lamp by a water screen. In making the observations one observer manipulated the cage by inverting it for each start and then noted the instant that six beetles (50 per cent of all) reached the top of the cage. The start and stop were signalled to the second observer who kept time with the stop-watch and recorded the time intervals to the nearest

TABLE II.*

I	$\log_e I$	Reaction time.	$R = \frac{100}{\text{Reaction time}}$	$R = \frac{\log_e I + K}{k}$
			Observed.	Calculated.
		sec.		
85	4.44	15.50	6.45	6.56
234	5.45	13.14	7.61	7.49
608	6.40	11.60	8.62	8.36
1,600	7.37	10.86	9.21	9.25
3,276	8.08	10.22	9.78	9.90

* I = the intensity of the light in candle meters, R = rate at which the organisms respond, k = 1.09, and K = 2.71.

second. Five observations were made at each intensity with each lot of twelve animals. Seven such series were run. Each point is therefore the result of thirty-five observations. The mean of the values obtained for each intensity is given under the heading "Reaction time" in Table II. The rate is equal to $\frac{100}{\text{Reaction time in seconds}}$.

We have assumed that the rate of movement is an objective measure of the effect of light on the organism, *i.e.* sensation, and now proceed to consider our experimental results in the light of the Weber-Fechner concept. According to Weber's law, the least noticeable difference of a stimulus is proportional to the magnitude of the pre-existing stimulus. Fechner[1] has expressed this relation in the form

[1] Fechner, G. T., Elemente der Psychophysik, Leipsic, 1860, i, 211.

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of a differential equation according to which $dS = c.\frac{dI}{I}$, in which S = the magnitude of sensation, I is the intensity of the exciting cause, and c is the constant of the series. Integrating, this equation becomes $S = c.\log_e I + K$. This means that the sensation is proportional

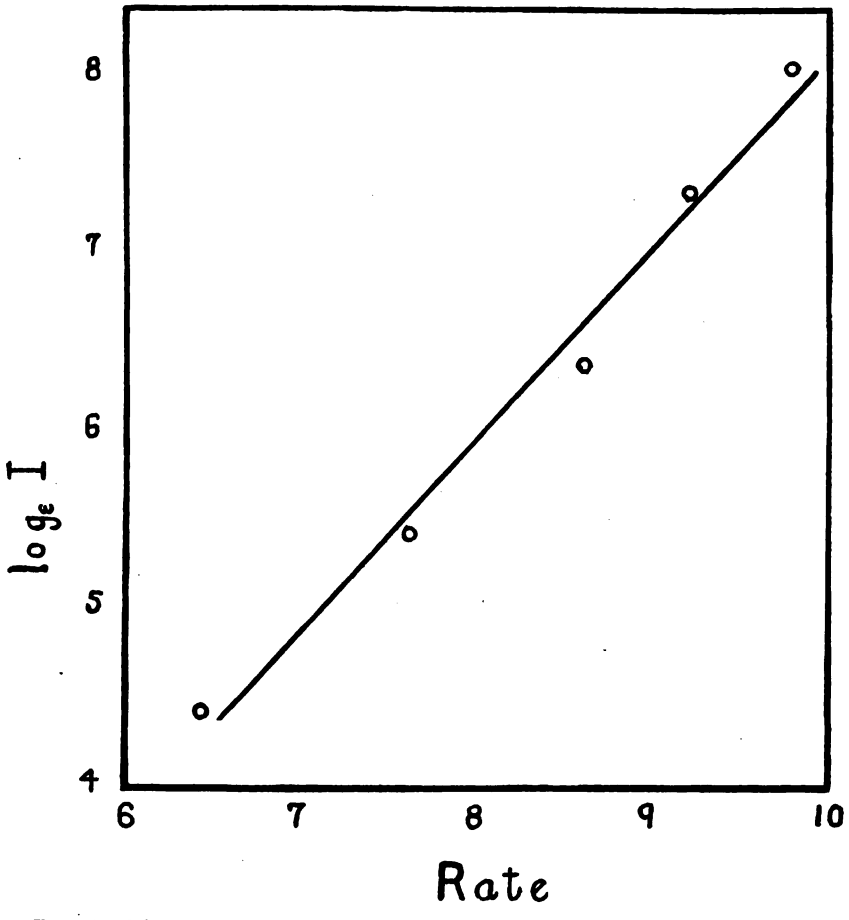

FIG. 1. The small circles show the experimental points in which Rate = $\frac{100}{\text{Reaction time in seconds}}$; the line is drawn through the points calculated for the rate by means of the equation, Rate = $\frac{\log_e I + K}{k}$.

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to the logarithm of the intensity of the stimulus. Putting R, the rate of response, for S, transposing and putting k for $\frac{1}{c}$, the integrated equation of Fechner becomes $\log_e I = kR - K$.

Substituting the experimental values of $\log_e I$ and of R in this equation and solving by the method of least squares for k and K we obtain $k = 1.09$ and $K = 2.71$. These quantities when put into the equation for each value of $\log_e I$ in turn yield a series of calculated values for R, as shown in the last column of Table II.

When the rates are plotted as abscissæ against the values of $\log_e I$ as ordinates the result is a straight line (Fig. 1), showing at a glance that the rate of response of the organism to light is proportional to the logarithm of the intensity of the illumination. This demonstrates that the Japanese beetle responds to light in accordance with the Weber-Fechner law.

SUMMARY.

Light and a temperature above 23°C. are necessary for the activity of *Popillia*.

The effect of light as indicated by the rate of locomotor response is related to light intensity according to Fechner's expression of Weber's law.

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COMPARATIVE STUDIES ON RESPIRATION.

XIV. Antagonistic Action of Lanthanum as Related to Respiration.

By MATILDA MOLDENHAUER BROOKS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, July 8, 1920.)

In previous papers[1,2] the writer has discussed the relation between respiration and antagonism, including antagonism between monovalent cations and bivalent cations. The present paper extends these studies to trivalent cations.

The experiments were made upon *Bacillus subtilis*. The method of experimentation has been fully described in previous papers.

The results are shown in the figures. Fig. 1 shows the manner in which the rate of respiration changes under the influence of $La(NO_3)_3$ in concentrations of 0.000006, 0.000025, 0.00005, 0.05, and 1.0 M. During the first 10 minutes the bacteria are under normal conditions and the curve (broken line) is horizontal. After this (at the point marked 0 on the abscissa) the salt is added. The addition of sufficient $La(NO_3)_3$ to make the concentration 0.000006 M produces an increase in the rate which remains constant during the period of experimentation. When the concentration is 0.000025 M the rate is normal, while in higher concentrations there is a decrease in rate which amounts almost to a cessation of respiration at 1 M. These curves are selected from a number of similar typical curves and each represents one experiment.

Fig. 2 shows the effect of various concentrations of $La(NO_3)_3$ upon the rate of respiration. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. There is an increase in the rate of production of CO_2 at 0.000006 M and a decrease in the rate at concentrations higher than 0.000025 M. As the effect

[1] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.

[2] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 331.

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of the lower concentrations cannot be clearly shown in the figure the results are given in Table I.

Fig. 3 shows antagonism. In this figure the abscissæ represent the number of cc. of each component in the mixture; since the molecular concentrations are the same for all the components the abscissæ

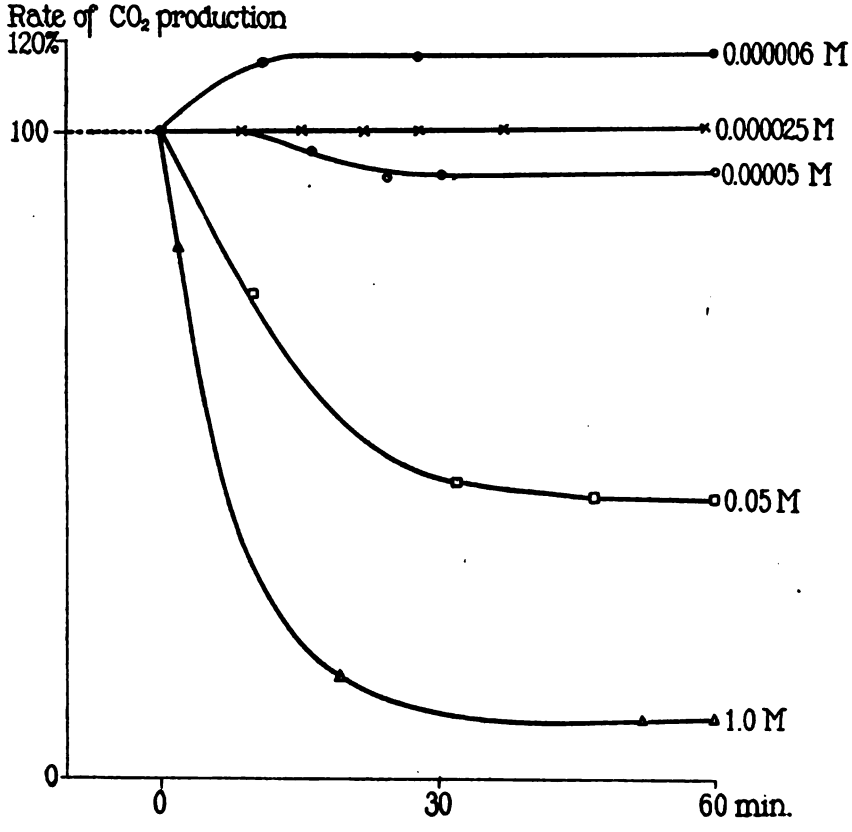

FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.000006, 0.000025, 0.00005, 0.05, and 1.0 M $La(NO_3)_3$. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each experiment is represented by a single curve.

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represent molecular proportions. The ordinate in the center represents the rate of respiration in a mixture of 50 cc. of each component; the ordinate at the extreme left represents the rate in a pure solution of one component; the ordinate at the extreme right, the rate in a

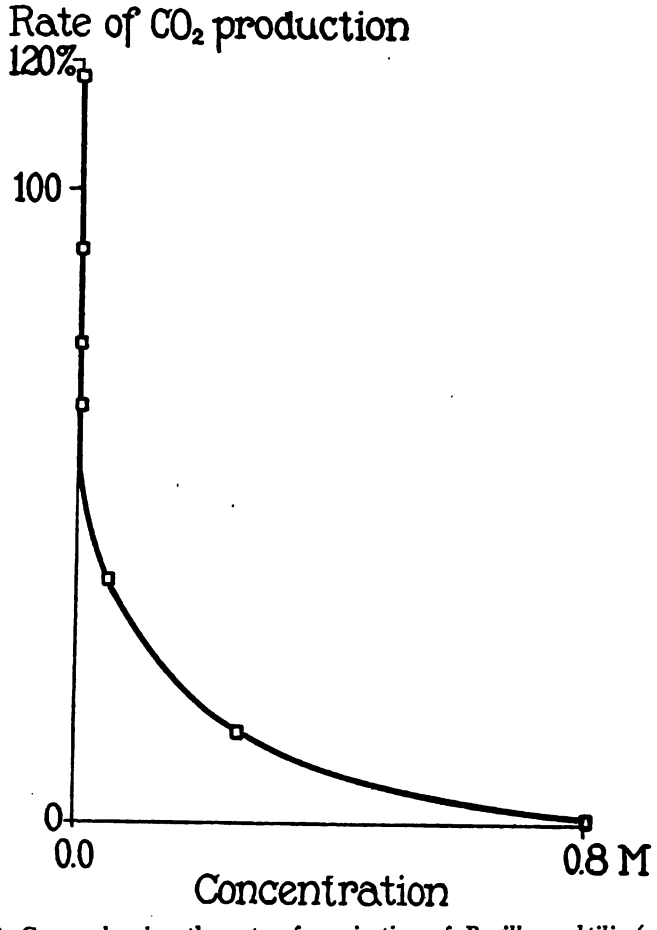

FIG. 2. Curve showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as affected by $La(NO_3)_3$. The normal rate represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Average of three or four experiments; probable error of the mean less than 2 per cent of the mean.

Table I accompanies Fig. 2, showing the results at concentrations too small to be represented properly in the figure.

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pure solution of the other component. The arrangement is made clear in the legend to the figure in each case by placing the words "left" and "right" after the component. Thus Curve A shows antagonism between NaCl (left) and $La(NO_3)_3$ (right) in concentrations of 0.8 M. A very small amount of $La(NO_3)_3$ sufficed to antagonize NaCl, the optimum proportion being 99.8 parts of NaCl to 0.2 parts of $La(NO_3)_3$.

Since in a previous investigation[1] it was found that one part of $CaCl_2$ was required to antagonize five parts of NaCl, it is evident that La is much more effective than Ca in antagonizing Na. This accords with the results of experiments by other investigators on

TABLE I.

*Rate of Respiration of Bacillus subtilis in Various Concentrations of Lanthanum Nitrate.**

Molecular concentration of $La(NO_3)_3$...	0.8	0.25	0.05	0.005	0.0005	0.00005	0.000025	0.0000060	0.0000025
Rate of CO_2 production.....	0	21	38	66	76	91	100	117	100

* Each number (representing the average of three or four experiments) expresses the rate (calculated as per cent of the normal) after an exposure of 1 hour. Probable error of the mean less than 3 per cent of the mean.

growth, length of life, electrical conductivity, etc. in which it is found that trivalent cations are more effective than bivalent in antagonizing monovalent cations.[3]

Curve B shows the effects of combinations of $CaCl_2$ (left) and $La(NO_3)_3$ (right) in concentrations of 0.8 M. There is a slight amount of antagonism which attains a maximum at 8 parts of calcium and 2 parts of lanthanum; from this point the curve approaches rapidly zero as the amount of lanthanum is increased.

[3] *Cf.* Loeb, J., The dynamics of living matter, New York, 1906. Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 464.

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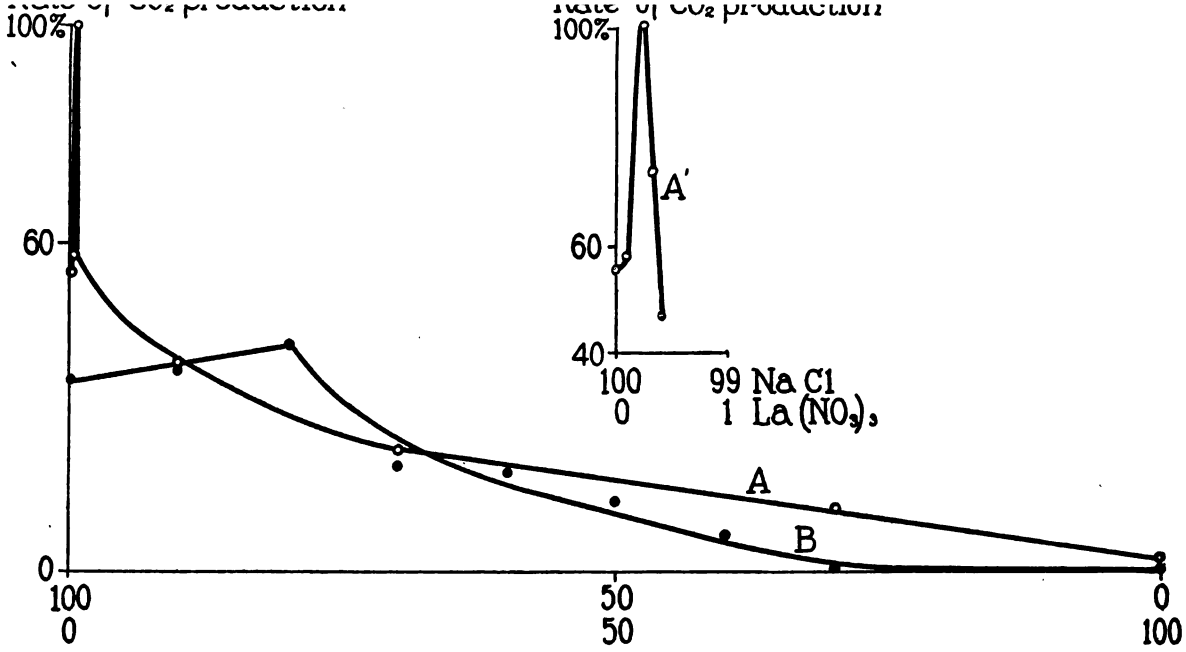

FIG. 3. Curve A shows antagonism between NaCl, 0.8 M (left) and $La(NO_3)_3$, 0.8 M (right). A portion of Curve A is represented on an enlarged scale (as A′) in the upper part of the figure. This was necessary because the ratios of the two salts in this region of the curve could not be shown accurately in Curve A.

Curve B shows a slight amount of antagonism between $CaCl_2$, 0.8 M (left) and $La(NO_3)_3$, 0.8 M (right).

The ordinates represent the rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the salts used. Thus in Curve A, the ordinate at the extreme left represents the rate in 0.8 M NaCl, while the ordinate at the extreme right represents the rate in 0.8 M $La(NO_3)_3$. The ordinate in the middle represents the rate in 50 parts of 0.8 M NaCl and 50 parts of 0.8 M $La(NO_3)_3$. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in about 30 seconds, varying according to the number of bacteria used. Curve A, average of three experiments; probable error of the mean less than 2 per cent of the mean. Curve B, a single typical experiment.

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SUMMARY.

1. Concentrations of $La(NO_3)_3$ up to 0.000025 M have little effect upon the rate of respiration of *Bacillus subtilis*; at 0.000006 M there is an increase in rate, while in higher concentrations there is a decrease in rate.

2. There is well marked antagonism between $La(NO_3)_3$ and NaCl, and very slight antagonism between $La(NO_3)_3$ and $CaCl_2$.

3. It requires a very small amount of $La(NO_3)_3$ to antagonize NaCl, the proportions of the two salts at their maximum antagonism being 99.8 parts of NaCl and 0.2 parts of $La(NO_3)_3$.

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ON THE DIFFERENTIATION OF THE LEAF TISSUE FLUIDS OF LIGNEOUS AND HERBACEOUS PLANTS WITH RESPECT TO OSMOTIC CONCENTRATION AND ELECTRICAL CONDUCTIVITY.*

By J. ARTHUR HARRIS, ROSS AIKEN GORTNER, and JOHN V. LAWRENCE.

(From the Department of Experimental Evolution and the Department of Botanical Research, the Carnegie Institution of Washington, Washington.)

(Received for publication, October 30, 1920.)

The existence of a differentiation of ligneous and herbaceous plants with respect to the magnitude of the osmotic concentration of the tissue fluids was first demonstrated in a strictly quantitative manner by work on the sap of the plants of the spring flora of the Arizona deserts[1] in the neighborhood of the Desert Botanical Laboratory, and on the terrestrial vegetation of the Jamaican montane rain forest.[2] These studies, in two geographically widely separated and climatically dissimilar regions, and an extensive series of unpublished observations demonstrate that the leaf tissue fluids of ligneous plants are characterized by an osmotic concentration materially higher than that of herbaceous forms.

The magnitude of the specific electrical conductivity, K, of the fluids must now be considered in comparison with osmotic concentration as measured by the freezing point lowering, Δ, for a series of plant species on which both of these constants were determined.

The determinations here considered were made on the north shore of Long Island during the spring and summer of 1914 and 1915. Leaf

* Studies carried out by the cooperation of the Department of Experimental Evolution and the Department of Botanical Research of the Carnegie Institution of Washington. The results will be published in full in the *Journal of Physical Chemistry*.

[1] Harris, J. A., Lawrence, J. V., and Gortner, R. A., *Phys. Researches*, 1916, ii, 1.

[2] Harris, J. A., and Lawrence, J. V., *Am. J. Bot.*, 1917, iv, 268.

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tissue was collected in large test-tubes and squeezed as completely as possible after freezing to render the tissue permeable as has been shown to be necessary by Dixon and Atkins and by ourselves. The freezing point lowering, Δ, was determined in the usual manner. Correction was made for the ice separating on undercooling. The specific electrical conductivity, K, of the centrifuged sap was measured at 30°C. in a Freas conductivity cell, standardized by considering the conductivity of 0.1 N KCl to be 0.01412 reciprocal ohms, by means of the ordinary meter bridge wire.

The determinations for each species were then averaged and the deviation of each determination from the average for the species was calculated. All numbers which showed a deviation of more than ±20 per cent for either Δ, K, or $\frac{K}{\Delta}$ were discarded, and a new average with

TABLE I.

Growth form.	Average. Δ	Average. $K \times 10^6$	Average. $\frac{K}{\Delta} \times 10^6$
Trees	1.292±0.043	11,213±494	9,092±462
Shrubs	1.177±0.024	10,770±339	9,529±372
Trees and shrubs	1.217±0.022	10,922±281	9,378±292
Herbs	0.846±0.011	14,308±192	17,674±282

deviations $< \pm 20$ per cent was determined. The statistical constants are, therefore, based on carefully selected averages whenever more than a single determination was available for a species. Determining the averages from the protocols of measurements we have the results for the three growth forms, and for a combination of the two groups of ligneous plants set forth in Table I.

The constants show that the tissue fluids of both trees and shrubs are characterized by a far greater freezing point lowering than are those of herbaceous plants. The mean freezing point lowering of the leaf tissue fluids is greater, although perhaps not significantly greater in comparison with its probable error, in arborescent than in shrubby species. The differences between trees and herbs, shrubs and herbs, and all ligneous plants and herbs are several times as large as the probable error of the difference and so unquestionably significant.

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Expressing the differences in per cents of the values for ligneous forms, we note that the value for trees and shrubs is 30.46 per cent higher than that of herbaceous plants. These results are, therefore, in excellent agreement with those found in the Arizona deserts and in the Jamaican rain forest.

The constants for specific electrical conductivity show that the conductivity for shrubs is slightly lower than that for trees. The difference is, however, smaller than its probable error. The differences between the conductivities of the fluids of trees and herbs, shrubs and herbs, and both ligneous forms and herbs are several times as large as their probable errors and show that the conductivity is distinctly higher in herbaceous than in ligneous species.

The ratio of conductivity to freezing point depression is much smaller in both trees and shrubs than it is in herbs. The mean value of $\frac{K}{\Delta}$ is lower in trees than in shrubs, although the difference cannot be considered significant in comparison with its probable error. The ratios ($\times 10^6$) are 9,092:17,674 in the case of trees and herbs and 9,529:17,674 in the case of shrubs and herbs. Since the ratio does not differ significantly in trees and shrubs it is quite proper to combine them, and we obtain an average of $\frac{K}{\Delta}$ in all ligneous plants of 9,378 $\pm$ 292 as compared with 17,674 $\pm$ 282 in herbs. Thus the ratio $\frac{K}{\Delta}$ is about 90 per cent higher in herbaceous than in ligneous plants.

The foregoing results show clearly that the osmotic concentration is higher while the electrical conductivity is lower in the tissue fluids of ligneous than in those of herbaceous species. Because of the wide geographic range and the great diversity of conditions (xerophytic, mesophytic, and hygrophytic) under which the investigations on osmotic concentration were carried out there can be no reasonable doubt that the differentiation of ligneous and herbaceous plants with respect to the magnitude of their osmotic concentration is a general biological law. Until confirmed by investigations in other regions presenting different conditions for plant growth—investigations which are now in progress—the results for conductivity cannot be asserted to be of universal validity.

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EXPERIMENTAL PRODUCTION OF GIGANTISM BY FEEDING THE ANTERIOR LOBE OF THE HYPOPHYSIS.

By EDUARD UHLENHUTH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 29, 1920.)

Perhaps the most reliable information as to what may be the function of the hypophysis (or any other endocrine gland) may be expected to be obtained through experiments on the extirpation and transplantation of the gland. The majority of these experiments have shown in a rather conclusive way, that growth and development are inhibited in the partial (mammalians) or total (amphibians) absence of the anterior lobe; an increase of the rate of growth ensues if anterior lobes are grafted to the animals.[1] Particularly clear are the results obtained in amphibians. As shown by Smith[2] and by Allen,[3] the extirpation of the anterior lobe of the hypophysis results in an inhibition of growth and metamorphosis of the operated tadpoles. Recently Allen[4] has shown that grafting the anterior lobe of adult frogs on tadpoles causes an acceleration of growth and development in normal larvæ, and that it also restores the power of growth and development after they had been lost through extirpation of the anterior lobe.

These experiments seem to demonstrate that the anterior lobe of the hypophysis is the organ which makes growth possible during the normal growth period of life. They do not afford, however, any evidence as to whether the substance of the anterior lobe can cause growth to continue beyond the period of life in which, under normal conditions, the ability of growth is lost and whether in this way the anterior

[1] For a more complete discussion of the literature see Uhlenhuth, E., The rôle of the internal secretions in growth and development, in a book on Internal secretion and metabolism, edited by L. F. Barker and R. G. Hoskins (in press).

[2] Smith, P. E., *Science*, 1916, xlix, 280; *Anat. Rec.*, 1916–17, xi, 57.

[3] Allen, B. M., *Science*, 1916, xliv, 755.

[4] Allen, B. M., *Science*, 1920, lii, 275.

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lobe substance can increase the size of the individual over the normal "maximum" size of the species. It is well known that growth of every individual stops as soon as the specific size of the species is reached. Many problems pertaining to this phenomenon would appear in a new light if it were possible to cause gigantism by a particular substance.

Clinical observations point to the conclusion that at least one form of gigantism is caused by an excessive production of anterior lobe substance; nevertheless, attempts to produce experimental gigantism have so far been unsuccessful. The only way to attack this problem seems to be the feeding of the anterior lobe substance by mouth. Such experiments have been attempted in large numbers but the results have for the most part been contradictory and difficult to interpret. The majority of investigators have merely desired to determine whether or not feeding of anterior lobe modifies in a specific way the rate of growth. It will be pointed out later that the greatest care is necessary in the interpretation of results obtained from feeding experiments. From the more recent feeding experiments, and especially those performed by Hoskins and Hoskins[5] and by Smith[6] on tadpoles, by Robertson[7] and his coworkers on white mice, and by Wulzen[8, 9] on chickens, most students of endocrinology have concluded that the anterior lobe substance retards growth in early periods of life, while later on it may cause an acceleration of growth.

But in these experiments none of the animals fed with anterior lobe developed into giants, except in two cases in which the slightly greater size of the experimental animals may have been due to the effect of the anterior lobe substance. Robertson and Ray[10] claim that they obtained unusually large mice, when the feeding of anterior lobe substance was started at an age of 4 weeks and discontinued at an age

[5] Hoskins, E. R., and Hoskins, M. M., *Endocrinology*, 1920, iv, 1.

[6] Smith, P. E., *Univ. California Pub., Physiol.*, 1918, v, 11.

[7] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385, 397, 409. Robertson, T. B., and Delprat, M., 1917, xxxi, 567. Robertson, T. B., and Ray, L. A., 1919, xxxvii, 393, 427, 455.

[8] Wulzen, R., *Am. J. Physiol.*, 1914, xxxiv, 127.

[9] Wulzen, R., *J. Biol. Chem.*, 1916, xxv, 625.

[10] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 455.

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of 12 weeks. In experiments in which the chicks were fed anterior lobe from an early stage, Wulzen[9] succeeded in raising one anterior lobe-fed cock which weighed 1,882 gm. as against the weight of only 1,597 gm. of the control animal; no normal cock raised by Wulzen grew to the size of the hypophysis-fed bird. Wulzen, however, raised only one cock of this kind; moreover, no records are given in the paper as to the normal maximum size of that race of chickens, and, therefore, it is not certain that this cock could be actually considered a true giant.

From the experiments to be reported in this paper, it will become evident that at least in one group of cold blooded animals, namely in salamanders, feeding of anterior lobe leads to the attainment of a size considerably in excess of not only the normal "average" size, but of even the greatest known size of the two species (*Ambystoma opacum* and *Ambystoma tigrinum*) employed in these experiments.

Critical Remarks Concerning the Methods of Feeding.

It is usually stated that in order to obtain reliable results in feeding experiments of this kind, only small amounts of the glandular product should be added to an otherwise normal diet. I, however, am of the opinion that this method, although considered at present as the standard method, cannot, at least under all conditions, give conclusive results. It is possible that a gland may contain a specific growth-promoting principle and yet may not give, with the above mentioned method, positive results if the amount of the hormone fed is too small. We do not know how much of a supposedly hormonic substance is contained in a definite amount of the fresh product, nor do we know how much of it may be required to produce gigantism or accelerated growth. While most hormones act in small quantities, it would be premature to conclude that all hormones should act in small amounts. The main point in experiments of this kind is to be able to prove that the control animals are fed in such a way as to show the maximal rate of normal growth known to exist in the species experimented upon.

In my experiments the controls were fed exclusively the normal diet, while the experimental animals were fed exclusively on the anterior lobes of the hypophysis of cattle. This method is based on the

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following considerations. If earthworms are a complete diet for salamanders, the worm-fed animals should grow at the maximum rate characteristic of the species, provided that the calories and vitamines are in sufficient quantity to make the maximum rate of growth at that particular temperature possible. If by chance the anterior lobe should have a higher food value than the earthworm, this could not result in a better growth as long as the food value of the earthworm suffices for the maximum normal growth. If the anterior lobe of the hypophysis contains only ordinary food substances and no specific hormone for growth, the rate of growth of the animal should not exceed the specific maximum rate.

Hence only two conditions had to be provided for. First, all animals, controls and experimental, had to be kept at the same temperature, a condition which was carefully provided for. Second, all animals were given as much food as they desired to take. This condition was approached as nearly as possible.

The worm-fed animals took the worms readily and voluntarily. The gland-fed animals, however, had to be fed by pushing the pieces of gland into their mouths; yet it was possible to adjust the quantity of food according to the appetite of the animals. They yield readily if the food is gently pressed against their jaws, and swallow the food quickly when they are hungry; while they push the food back by means of the tongue or eject it when it is forced into the mouth, if they are not hungry.

The quantity of food taken up by the controls was generally greater than the quantity of hypophysis taken by the experimental animals.

Characteristics of Growth of Normal Salamanders.

Two species of salamanders were employed in these experiments, *Ambystoma opacum* and *Ambystoma tigrinum*. Since little is known about the normal growth of these animals, some data pertaining to it may be recorded here.

Large numbers of *Ambystoma opacum* were reared from eggs in my laboratory and the growth of the species has been recorded over long periods. The longest record I possess is that of four animals which at present are 3 years (161 weeks) of age and were raised from eggs of

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two different females. Many animals of this species were observed for a period of over 2 years and behaved essentially the same way. Fig. 1 shows the growth curves of the four 3 year old animals and illustrates the most important characteristics of the growth of that species. Although each animal, during the larval period, was treated in a different way (D_1 normal, E_3 underfed, $W_{Ca}5$ kept in water + a small amount of Ca lactate, $W_{Na}4$ kept in water + a small amount of Na lactate) all four curves are about the same. The most rapid growth takes place during the larval period (first period of growth); at the end of this period a decrease in size is noticeable which may last for several weeks and corresponds to metamorphosis. The second period of growth lasts from after metamorphosis till the first breeding season, at which the animals are about 1 year old. The beginning of the breeding season in the male may be recognized by the swelling and reddening of the cloacal region and in both males and females by the cessation of food intake. During the second period of growth the animals continue to grow at a fairly high rate; during the first as well as the following breeding seasons little growth or even a decrease in size may be noticed. The third period of growth begins after the first breeding season and lasts till the third breeding season, and is characterized by slow growth. No records are available to determine the behavior after the third breeding season, but to conclude from the normal maximum size of the species growth seems to be nearly completed at the end of the third period. The control animals of the hypophysis-fed series exhibit a similar type of growth (Fig. 2), although they were from a different season (1918). It is safe to assume that this is the type of growth characteristic for the species *Ambystoma opacum*. The most important feature of it, in connection with the following experiments, is the considerable slowing down of growth after the first breeding season.

As to the normal size of the species, the following records are available. The largest animal on record in my laboratory was 115 mm. long at an age of 79 weeks (it was one of the controls of the hypophysis-fed animals). The largest animal among fifteen specimens of from 2 to 3 years of age, still alive at present, measures 113.5 mm., the average size of these fifteen animals being 103.5 mm. (the small size of some of them may be due to the abnormal conditions under

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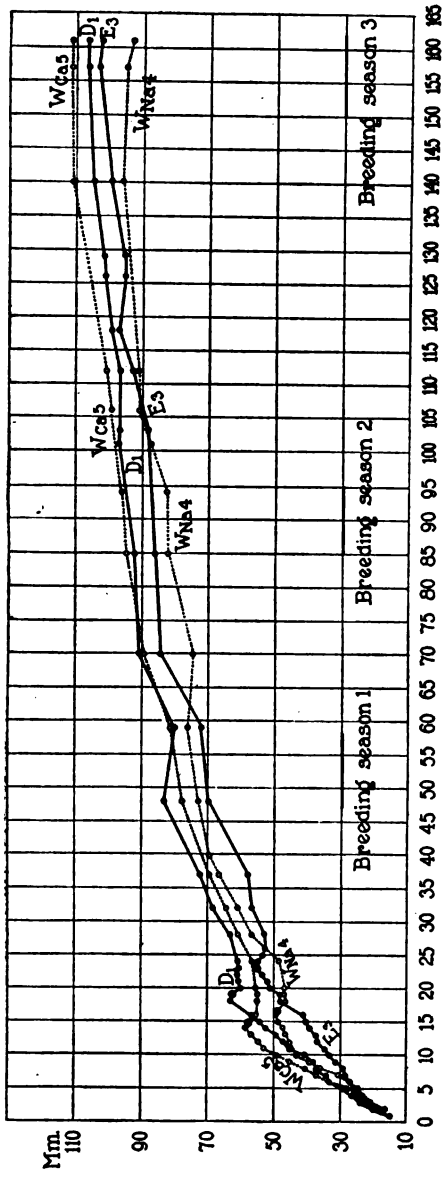

FIG. 1. Growth of four specimens of *Ambystoma opacum* during a period of 3 years. All four animals were fed normal diet, but E_3 was underfed during the larval period and W_{Ca} 5 and W_{Na} 4 were kept in water + small amounts of Ca lactate and Na lactate respectively during the larval period. The figure is drawn to a smaller scale than Figs. 2, 3, 4, and 5.

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which they were kept during the larval period). Two breeding females, collected recently outdoors, measured 112 mm. and 106 mm. respectively.[11] Cope,[12] in his book on North American batrachians, mentions the specimen, from which he described the species, as measuring 3.8 inches (about 100 mm.). The largest animal found in the collection of the American Museum of Natural History[13] measured 117.7 mm.; the average of the eight largest animals was 100.3 mm.

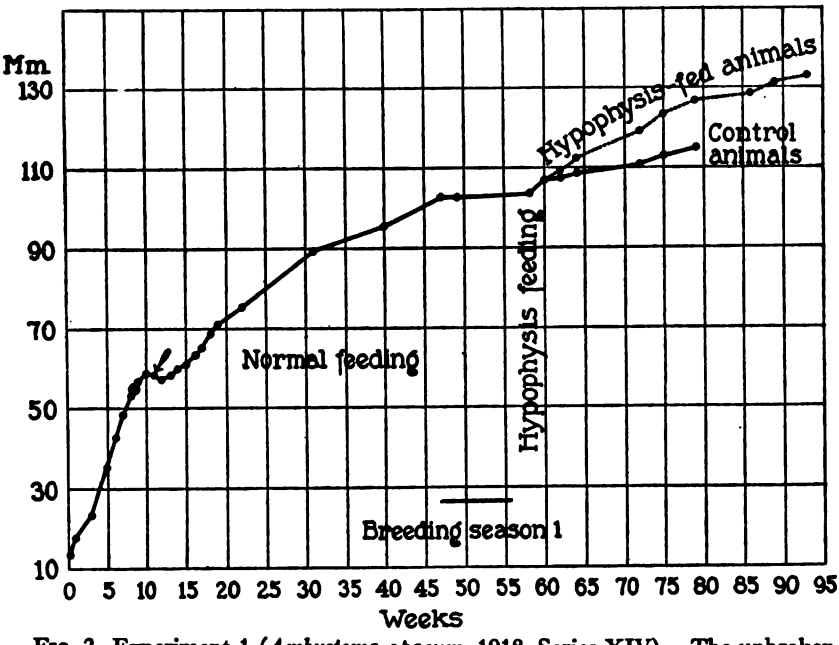

FIG. 2. Experiment 1 (*Ambystoma opacum*, 1918, Series XIV). The unbroken line indicates the growth of the controls, the dotted line that of the anterior lobe-fed animals.

To conclude from these data the maximum size of the species is nearly 118 mm., while specimens measuring more than 115 mm. are of very rare occurrence.

[11] I am obliged to Mr. George P. Engelhardt, of the Brooklyn Museum, for this record.

[12] Cope, E. D., The batrachia of North America, Washington, 1889.

[13] I wish to express my appreciation to Miss M. Dickerson for giving me the opportunity of examining the collection of salamanders at the American Museum of Natural History.

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I have no other records concerning the growth of the species *Ambystoma tigrinum* except those afforded by the control animals of these experiments. These give information on the growth during a period of only 1½ years (84 weeks), the present age of the animals of Series A, XLVI, and LV. Apparently this species, as illustrated in Figs. 3, 4, and 5, behaves very much in the same way as the species *Ambystoma opacum*, growth being most rapid during the larval period, and continuing at a fairly high rate till about the end of the 1st year, after which it becomes rather slow.

It is not so simple to determine the maximum size of this species as in the case of *Ambystoma opacum*, since there are two races of the tiger salamander, a western and an eastern one, which are very different in respect to the type of growth. The western race seems to be naturally a giant race; this is true at least for the specimens which metamorphose from the well known neotenous larvæ found in the western lakes. This condition, however, is doubtlessly caused by disturbances of the endocrine system and is rather pathological than normal. My experiments, performed with eastern animals, cannot be checked by means of these giants although they are apt to throw some light on the gigantism of the neotenous specimens and those that have metamorphosed from neotenous larvæ. Cope in describing a large number of specimens of *Ambystoma tigrinum* mentions that the largest specimen among them measured 10 inches (about 244 mm.) and that De Kay described a still larger one measuring 11 inches (about 280 mm.). The largest specimen among 55 neotenous larvæ which I myself collected in the vicinity of Tolland, in the Colorado Rocky Mountains, measured 257 mm., while all the metamorphosed specimens collected in the same locality were much smaller. As pointed out above, I have used in my experiments only eggs that were from females of the eastern race. Unfortunately, I have not reared enough animals of this species to form a conclusive idea as to the normal maximum size of the species. But the largest individual on record in my laboratory (one of the controls of these experiments) measures 200 mm. at an age of 84 months; it is, however, still growing, although very slowly. In the collection of the American Museum of Natural History I found the largest specimen among the nine largest animals, eastern as well as western, to measure 208.7 mm.

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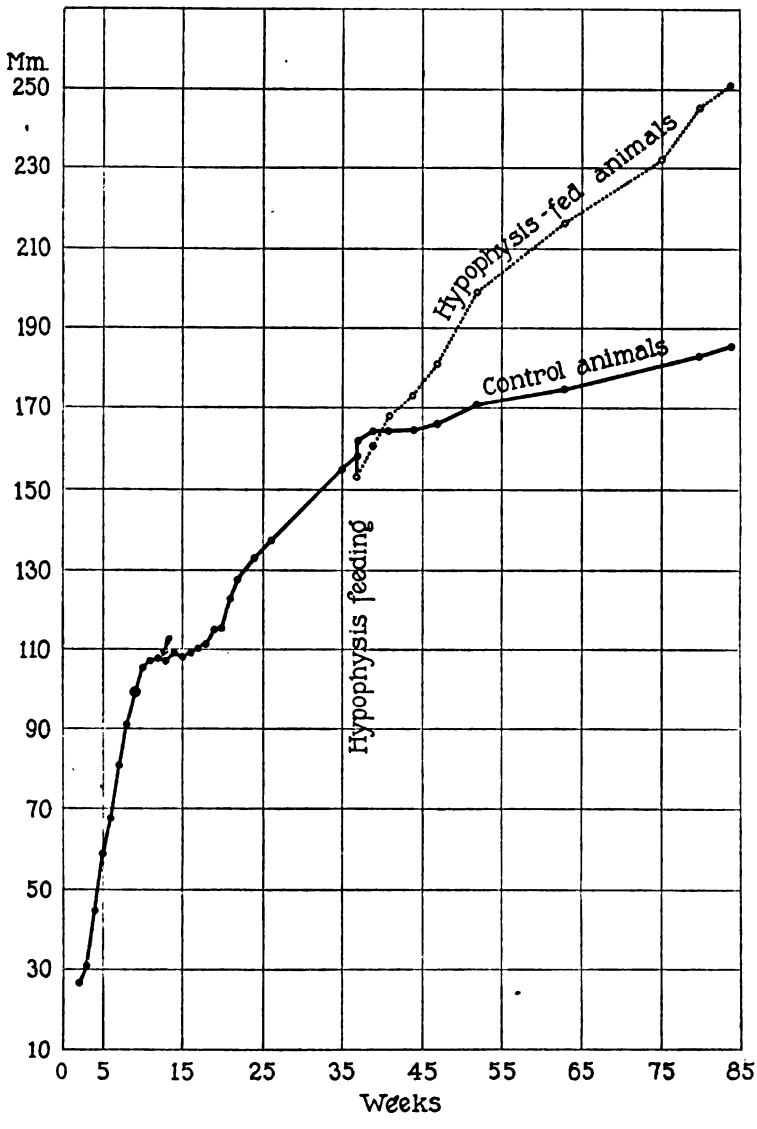

FIG. 3. Experiment 2 (*Ambystoma tigrinum*, 1919, Series XLVI). The unbroken line illustrates the growth of the controls, the dotted line that of the anterior lobe-fed animals.

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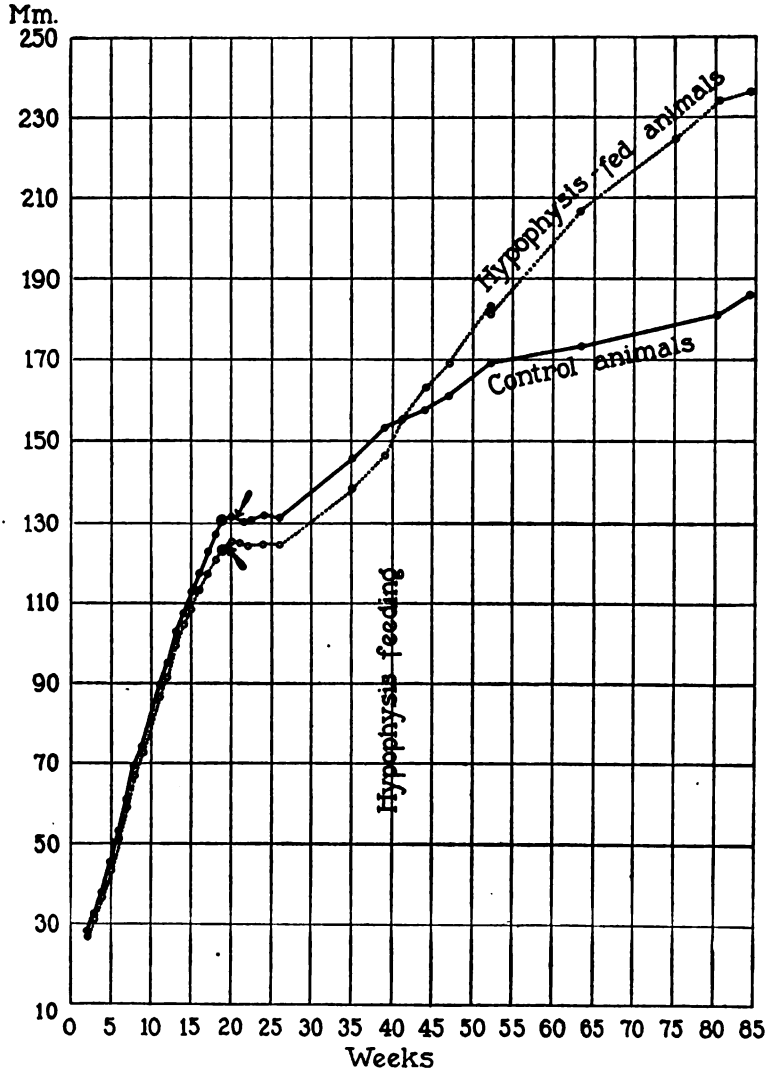

FIG. 4. Experiment 3 (*Ambystoma tigrinum*, 1919, Series LV). The unbroken line represents the growth of the controls, the dotted line that of the anterior lobe-fed animals.

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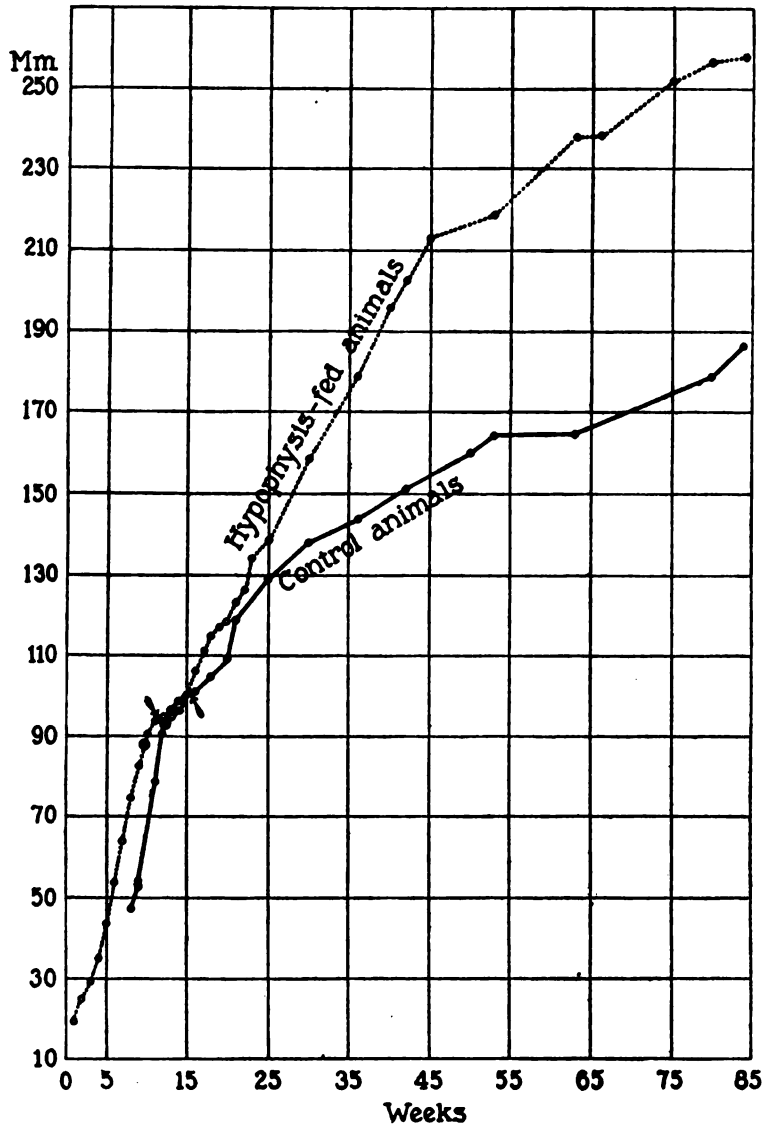

FIG. 5. Experiment 4 (*Ambystoma tigrinum*, 1919, Series A and XXXVII). The unbroken line represents the growth of the controls, the dotted line that of the anterior lobe-fed animals.

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while the average was 200.8 mm. From these data it seems that while the largest specimen that could be found among eastern animals of this species measured 208.7 mm., pathological conditions of the endocrine system may produce giants of a maximum size of 280 mm.

The Growth of the Hypophysis-Fed Animals.

Experiment 1.—Of four normal metamorphosed specimens (Series XIV) of *Ambystoma opacum* which had been reared in the laboratory from eggs of the same female (Brood 1918) two were started on a diet of anterior lobe of cattle hypophysis at an age of 60 weeks, *i.e.* at the beginning of the third period of growth; the other two animals were controls and were kept on an abundant diet of earthworms. The size of the animals at the beginning of the experiment may be seen from Table I.

TABLE I.

Hypophysis-Fed Ambystoma opacum, Experiment 1.

	Animal No.	Size of animals.		
		60 weeks.	72 weeks.	End of experiment.
		mm.	mm.	mm.
Control	4	102.1	108.1	108.1 at 72 weeks.
	1	111.0	113.2	115.0 " 79 "
Anterior lobe-fed animals	2	110.4	120.6	130.5 " 105 "
	3	103.1	118.8	138.0 " 101 "

The rate of growth of the anterior lobe-fed animals, after 2 weeks of feeding, rose over that of the controls and continued at such a height as is usually observed only in the second period of growth (Fig. 2). The two control animals, although they were fed on an abundant diet, continued to grow at a slow rate (Fig. 2) characteristic of the third period of growth (Fig. 1). In consequence of their rapid growth the anterior lobe-fed animals, 12 weeks after the beginning of the experiment, had reached a size exceeding that of the largest animals of this species on record (Table I, 72 weeks). At the end of the experiments (caused by the death of the animals) the largest anterior lobe-fed animal measured 138 mm., the smaller one 130 mm., while the controls had reached a size of only 115 and 108 mm. respectively. Unfortu-

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nately both of the controls were lost before the anterior lobe-fed animals had reached such an extraordinary size, but from our experiences with many other animals as well as from the maximum size of this species it is safe to conclude that the growth of the controls in this experiment was nearly completed. On the other hand, the size of the anterior lobe-fed animals exceeded the normal maximum size of the species so much as to leave no doubt that both of them were giants. The size of the larger experimental giant exceeded the size of the largest normal animal of this species on record by 20 mm., and the size of the smaller experimental giant exceeded the largest normal animal on record by 12 mm. It is certain that both animals would have become still larger had they survived, since they were still growing before they died.

TABLE II.

Hypophysis-Fed Ambystoma tigrinum, Experiment 2.

	Animal No.	37 weeks.	63 weeks.		84 weeks.	86 weeks.
		Size.	Size.	Weight.	Size.	Weight.
		mm.	mm.	mg.	mm.	mg.
Controls.....	2	152.4	163.0	22,400	171.5	34,800
	3	163.5	183.0	31,200	190.0	40,000
	4	167.9	179.0	27,900	197.0	35,200
Anterior lobe-fed animals.....	5	163.5	233.0	57,200	268.0	72,300
	6	142.5	199.0	50,500	233.0	78,000

Similar results were obtained when metamorphosed specimens of the species *Ambystoma tigrinum* were fed on anterior lobe.

Experiment 2.—Five larvæ (Series XLVI) were reared from eggs of the same female and fed on earthworms. At the end of the second growth period, about 24 weeks after metamorphosis, when the animals were 37 weeks old, three of them, among them the largest one of the series, were kept on earthworms, while two, among them the smallest one, were started on an exclusive diet of anterior lobe (for initial sizes see Table II).

Although the control animals were fed on an abundant supply of earthworms, their rate of growth remained low as compared with that during the second period of growth. The animals fed on anterior

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lobe, however, commenced to grow so rapidly that their rate of growth soon exceeded that which prevailed even during the earlier period of life (see Fig. 3). At an age of 63 weeks, 26 weeks after the beginning of the experiment, the smallest animal fed on anterior lobe was 20 mm. larger than the largest control animal, although at the beginning of the experiment it was 10 mm. smaller than the smallest control specimen; its weight was more than twice the weight of that of the smallest control animal. At an age of 84 weeks the largest animal of this series fed on anterior lobe measured 268 mm. as compared with 197 mm., the latter being the size of the largest control animal. Hence the size of the largest anterior lobe-fed animal exceeds at present the size of the largest normal animal, on record in the laboratory, by 68 mm. and the size of the largest specimen of the eastern race of *Ambystoma tigrinum* by 60 mm. It is 12 mm. smaller than the largest specimen of the western race on record, which, however, cannot be considered normal, as pointed out above. Both the controls and the anterior lobe-fed animals are still growing and at the present time the rate of growth of the anterior lobe-fed specimens is still higher than that of the control animals (see Fig. 3). It is possible, therefore, that in a short time, the largest anterior lobe-fed animal will exceed in size even the largest western specimen known to the writer.

Experiment 3.—Six larvæ (Series LV), of the same brood from which the animals of Experiment 2 were taken, were raised on earthworms. At the end of the second period of growth, about 19 weeks after metamorphosis and at an age of 39 weeks, three of them (Nos. 1, 2, and 4), among them the largest one, were used as worm-fed controls; the other three animals, among them the smallest one, were kept on a pure anterior lobe diet (Table III). The largest of these latter animals died soon after commencement of the experiment.

The results were similar to those obtained in Experiment 2 and may be seen from Table III and Fig. 4. At an age of 84 weeks the larger one of the two hypophysis-fed animals measured 51 mm. more than the largest control animal, and 44 mm. more than the largest animal of the eastern race of the species known to the writer. The hypophysis-fed specimens as well as the control animals are still growing, but at present the growth of the anterior lobe-fed specimens of this series is less vigorous than that of the controls.

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Experiment 4.—In this experiment (Series XXXVII$_3$ and Series A) the controls were underfed and therefore cannot be used for comparison. Both the experimental as well as the control series were obtained from the eggs of the same female; but the control animals were underfed for several weeks during the larval period and the experimental animal, which was fed abundantly on anterior lobe from the beginning of its life, was kept in distilled water during the larval period, which tends to retard growth. The results are seen in Fig. 5. The anterior lobe-fed animal measured 258 mm. at an age of 84 weeks; *i.e.*, 58 mm. more than the largest normal animal kept in the laboratory and 50 mm. more than the largest specimen of the eastern race. At present, however, it is growing very slowly and less vigorously than the animals of Series A.[14]

TABLE III.

Hypophysis-Fed Ambystoma tigrinum, Experiment 3.

	Animal No.	39 weeks.	63 weeks.		84 weeks.	86 weeks.
		Size.	Size.	Weight.	Size.	Weight.
		mm.	mm.	mg.	mm.	mg.
Controls.....	1	163.8	182.2	27,400	200.2	38,500
	2	156.1	176.4	30,000	182.0	40,200
	4	137.2	161.5	22,500	176.5	31,100
Anterior lobe-fed animals.....	3	136.4	208.5	54,200	251.6	95,700
	5	146.7	204.0	60,000	221.5	84,300
	6	156.1	Dead.			

[14] Since this paper went to press, Dr. Leonhard Stejneger, head curator of the United States National Museum, was kind enough to go over the entire collection of the museum and let me have the measurements of the largest specimens of the species *Ambystoma opacum* and *Ambystoma tigrinum*. Since the measurements are based on a far larger collection of material than my own data reported in the preceding pages, they are of greater importance and will be added below. They confirm the opinion expressed in this article that feeding the anterior lobes of hypophysis produces gigantism in salamanders.

Ambystoma opacum.

Normal.	Hypophysis-fed.
mm.	mm.
119	138
116	130

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DISCUSSION.

The feeding of anterior lobe to metamorphosed salamanders has two different effects on the growth of the animals: first, it increases the rate of growth over that of normal animals; and, second, it maintains growth after the normal "maximum" size of the species has been attained. Have we any reason to consider these effects as the result of a "specific" growth-promoting hormone?

As to the first point, we cannot be quite certain that the rate of growth of our control salamanders was the highest rate of growth which can be obtained with a normal diet. Unfortunately the data available on the growth of metamorphosed salamanders are not numerous enough to decide if earthworms form a sufficiently complete diet for the two species in question. Although it is quite certain that these animals do not require a plant diet, not enough food materials have been tested by the writer to state on which food they nat-

The two largest hypophysis-fed specimens of *Ambystoma opacum* exceed in size the largest known animal of this species by 19 and 11 mm. respectively.

Ambystoma tigrinum.	
Eastern race.	
Normal.	Hypophysis-fed. (Age 88 weeks.)
mm.	mm.
235	273.5 (Experiment 2, No. 5)
223	235.5 (" 2, " 6)
	257.1 (" 3, " 3)
	226.2 (" 3, " 5)
	263.0 (" 4, " 2)

Among the five hypophysis-fed specimens of *Ambystoma tigrinum* three are considerably larger than the largest known specimens of the eastern race, the largest hypophysis-fed specimen exceeding in size the largest eastern specimen by 28.5 mm.

There is, as stated above, a western giant race of *Ambystoma tigrinum*. Dr. Stejneger gives 258, 265, 285, 285, and 292 mm. as measurements of the five largest specimens of this race. The largest of our hypophysis-fed animals, which has grown 5.5 mm. in the last 4 weeks and measures at the time of writing 273.5 mm., has already outgrown two of the western giants and will soon outgrow the three others if it continues to grow at the present rate.

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urally grow best. Feeding of calf thymus or posterior lobe of hypophysis of cattle does not induce any growth equal to that of worm-fed animals. In the feeding of salamander larvæ I have used many different substances (frog muscle, beef muscle, lymph gland, parathyroids, thymus, spleen, cheese, milk) with and without the addition of normal food; it is certain that in larvæ no other diet can produce a rate of growth higher than that produced by earthworms. But, of course, the metabolic processes involved in growth may be quite different for the larvæ and the metamorphosed animals.

On the other hand, if we look at the curves of the various series, it is noticeable that the curves for the worm-fed animals are very much like the normal growth curves of most other animals whose growth has been studied carefully. In particular they show the gradual flattening out of the growth curves of warm blooded animals. The curves of the anterior lobe-fed animals, especially those of *Ambystoma opacum* (Fig. 2), with their sudden rise above the flat level of the normal curve, differ from this general normal type.

Although at present we must postpone more definite conclusions, it seems at least probable that the rate of growth of the animals fed exclusively on anterior lobe is the result of a specific growth-promoting hormone contained in the anterior lobe of the hypophysis.

It is beyond doubt that the size of the hypophysis-fed animals exceeds the "maximum" size of the species. The animals fed on anterior lobe are true giants. The hormone of the anterior lobe is not only able to accelerate growth, but also—and this is of far greater importance—possesses the property of maintaining growth when the normal size of the species has been reached. The production of experimental gigantism by means of feeding anterior lobe proves that this organ contains actually a specific substance which can overcome the obstacles which are responsible for the discontinuation of growth when the normal size is reached.

These experiments do not give any clue as to whether the cells of the body are directly affected by the hormone or whether this hormone acts by the intermediation of another organ. In view of the results obtained with single cells, it is probable that the cells are not directly affected by the anterior lobe hormone. It was found by Shumway, by

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Chambers, and by Nowikoff[15] that the division rate of Protozoa is not increased if anterior lobe extract is added to the culture medium. The same is true for the cells of warm blooded animals. In as yet unpublished experiments Carrel found that in tissue cultures the growth of the cells of warm blooded animals cannot be accelerated if anterior lobe extract is added to the culture medium.[16] Our experiments show, however, that the cells of the organism are still capable of dividing at a time at which the organism as a whole has stopped to grow. Apparently the cessation of growth of the organism as a whole is not caused by a fundamental property of the cell to become incapable of growth, after growth has been going on for some time. This corroborates the well known tissue culture experiments of Carrel which would suggest that the cell protoplasm, if kept under proper conditions, can go on dividing for indefinite periods. Since the hypophysis-fed salamanders are still growing, it is impossible, at present, to say how long the anterior lobe hormone can maintain, within the organism as a whole, a condition permitting active growth of the cell. But it is possible to say that the size of the individual is not an obstacle to the further growth of an organism. It has frequently been claimed that the size of every species is determined by the mechanics and statics of the substances constituting the body of the organism. Although these principles may be among the reasons why the organism stops growing after it has attained a definite size, they cannot be the only reason; the occurrence of giants shows that at least a considerable increase in the specific size of the species would result if the size of the organism would be determined by the mechanic and static principles alone.

As pointed out above, the evidence that anterior lobe feeding can produce gigantism in warm blooded animals is not yet sufficient. It would be of great importance if it should be demonstrated that this difference is due to the difference in the body temperature of amphibians and warm blooded animals. Lenz[17] has recently called atten-

[15] Shumway, W., *J. Exp. Zool.*, 1917, xxii, 529. Chambers, M. H., *Biol. Bull.*, 1919, xxxvi, 82. Nowikoff, M., *Arch. Protistenk.*, 1908, xi, 309.

[16] I am indebted to Dr. Alexis Carrel for permission to quote these, as yet unpublished, experiments, which were carried out in Dr. Carrel's laboratory.

[17] Lenz, F., *Münch. Med. Woch.*, 1919, lxvi, 992.

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tion to the fact that in acromegaly the tendency to abnormal growth is confined to the prominent parts of the body, in which the temperature is lower than in the main body.

SUMMARY.

1. Metamorphosed salamanders of the species *Ambystoma opacum* and *Ambystoma tigrinum* were fed on a pure diet of the anterior lobe of the hypophysis of cattle; the controls were fed on an abundant diet of earthworms.

2. The rate of growth of the animals fed on the anterior lobe of the hypophysis was greatly increased over the rate of growth of normal animals.

3. Growth of the animals fed on anterior lobe did not cease after they had reached the normal "maximum" size of the species, and experimental giants were produced.

4. The largest animal of the species *Ambystoma opacum* fed on anterior lobe of the hypophysis was 19 mm. larger than the largest normal animal of this species known to the writer; the largest animal of the species *Ambystoma tigrinum* fed on anterior lobe is at present about 28 mm. larger than the largest normal animal of the eastern race of this species known to the writer.

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TIME AND INTENSITY IN PHOTOSENSORY STIMULATION.

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(Received for publication, November 11, 1920.)

I.

The photochemical effect of light depends upon its intensity and the time of its action. In previous studies I have investigated the separate effects of these two variables in their action on the photic responses of *Mya arenaria* (Hecht, 1918–19, *a*; 1919–20, *c*). I have also considered the interaction of these two factors under certain specially circumscribed conditions of particular interest (Hecht, 1919–20, *b*, *c*). In its more general bearings, however, the relation between the two variables has not been examined. This I propose to do now.

If the intensity of the stimulating light is kept constant and its time of action varied, it has been found (Hecht, 1918–19, *a*) that the photochemical effect (E) is a linear function of the time (t). This may be written

$$E = k_1 t \quad (1)$$

where k_1 is a constant of proportionality. On the other hand, if the time of action is kept constant and the intensity varied, the photochemical activity of the light is found (Hecht, 1919–20, *c*) to be directly proportional to the logarithm of the intensity (I). This may be expressed as

$$E = k_2 \log I \quad (2)$$

k_2 being a constant.

It follows from equations (1) and (2) that if both the intensity and the time are permitted to vary, the photochemical effect should be proportional to the product of the time of action into the logarithm of the intensity. In other words

$$E = kt \log I \quad (3)$$

the terms having their previous significance.

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If equation (3) is true experimentally, it should furnish a corroboration of the previous findings as expressed in equations (1) and (2). Moreover, it would then give substantial support from a new angle to the hypothesis which I have proposed to account for the photosensory behavior of *Mya* (Hecht, 1918–19, *a*). I have therefore undertaken a number of simple measurements which will give an unequivocal answer to the requirements of equation (3).

II.

An outstanding characteristic of the response of *Mya* to light is its reaction time. It has already been repeatedly shown in these studies on the photosensory process that this reaction time is composed of two parts, the sensitization period and the latent period (Hecht, 1918–19, *a*; 1919–20, *b*). The sensitization period represents the actual time of action (t) of the light in its relation to the reversible photochemical reaction

$$S \rightleftharpoons P + A$$

in the sense organ. The latent period is the portion of the reaction time during which the light is not required. It is occupied by the time taken for the secondary reaction

$$L \rightarrow T$$

to produce the amount of T necessary to initiate the nervous impulse which begins the response of the animals.

The velocity of the latent period reaction is directly proportional to the photochemical effect produced by the initial photochemical reaction. A constant photochemical effect gives a constant latent period. During ordinary stimulation of *Mya* by light, the latent period is constant and of minimal duration because the photochemical effect produced during the sensitization period is constant and maximal. The sensitization period, and consequently the reaction time, simply prolongs itself until the required accumulation of P and A is produced by the light.

The duration of the latent period cannot be decreased beyond this point because the velocity of the reaction

$$\mathrm{L} \rightarrow \mathrm{T}$$

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is maximal when the amount of P and A has been produced during the exposure occupied by the sensitization period. It is, however, possible to *increase* the latent period by exposing the animal for periods less than the sensitization period. Submaximal quantities of P and A are then produced and the latent period is prolonged, because the velocity of the latent period reaction is diminished. If merely a minimum exposure is given (presentation time of Laurens and Hooker, 1920) the amount of P and A produced is minimal, and the latent period is consequently very much prolonged. In any event, however, a given photochemical effect results in a constant duration of the latent period.

These findings have been secured by such a variety of methods, and have been demonstrated so frequently to investigators at Woods Hole, that it is somewhat surprising to have them questioned (Laurens and Hooker, 1920). The experiments to be described will furnish still another means of corroboration, sufficient, I hope, to disarm any criticism.

The photochemical effect of the light is maximal if it takes place during the sensitization period. An animal which is exposed to light indicates automatically when this maximum has been reached by the retraction of its siphon at the end of the reaction time. Because the latent period is constant, variations in the reaction time to lights of different intensities are the result of variations in the sensitization period. This then furnishes a simple method of testing equation (3). All that is required is to measure the reaction time of animals exposed to lights of different intensities.

III.

The intensity of illumination may be varied by placing the animal at different distances from a source of light. As sources of light I have used on different occasions a 40 watt, a 100 watt, and a 250 watt lamp. These are concentrated-filament Mazda lamps, and for our purposes may be considered as point sources. The intensity of illumination therefore varies inversely as the square of the distance from the source. The reaction time is measured with a stop-watch.

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Only dark-adapted animals are used; and between tests they are kept in complete darkness. At least 15 minutes are allowed between tests.

At different times during the summer I performed seven series of experiments: one with three animals; two with four animals; two with six animals; and two with seven animals. As an example, Fig. 1 gives the data of the series of experiments performed on July 28.

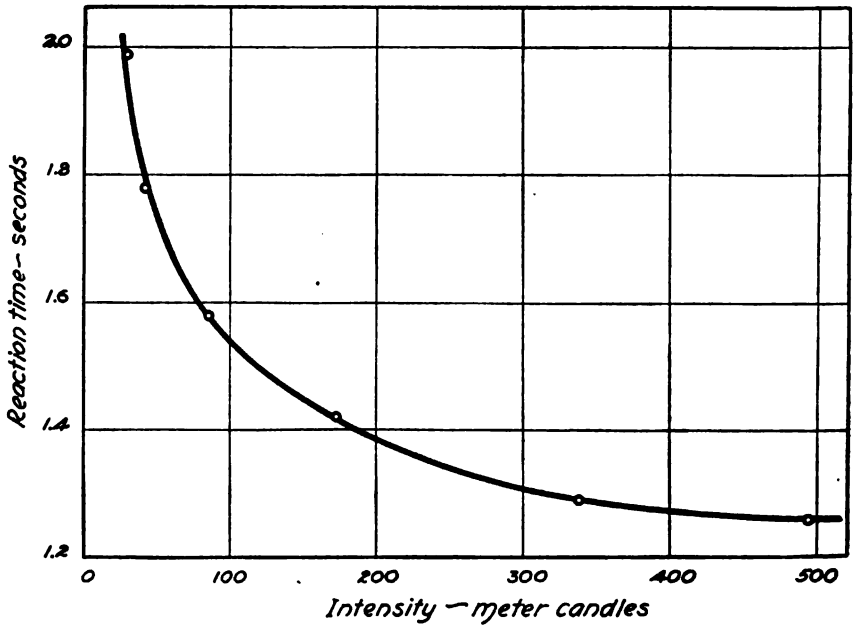

FIG. 1. Relation between the reaction time and the intensity of the stimulating light. Experiment 9.28; 40 watt lamp; six animals; three readings for each animal for every intensity.

The reaction time of each animal was measured at three different times for every intensity. Each point in Fig. 1 is thus an average of eighteen measurements, three with each of six animals. It is apparent that the experimental results are regular and may therefore be treated mathematically.

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IV.

Equation (3) states that

$$E = kt \log I \quad (3)$$

For purposes of experimental verification, and for convenience in presenting the data, it will be desirable to consider equation (3) as follows. The reaction time (r) is equal to the sum of the time occupied by the sensitization period (t) and the latent period (p). Therefore

$$r = t + p$$

and

$$t = r - p \quad (4)$$

Substituting this value of t in equation (3) we get

$$E = k(r-p) \log I$$

and from it that

$$r = K \frac{E}{\log I} + p \quad (5)$$

where $K = \frac{1}{k}$. It is at once apparent that equation (5) is in the form of the equation of a straight line

$$y = ax + b$$

where $y = r$, or the reaction time; $a = K$; $x = \frac{E}{\log I}$, which is equivalent to the reciprocal of the logarithm of the intensity, since E, the photochemical effect, is constant; and $b = p$, the latent period.

It follows from this consideration that if equation (3) is true, a curve, representing as ordinates the reaction time (r) and as abscissæ the reciprocal of the logarithm of the corresponding intensity $\left(\frac{1}{\log I}\right)$, should be a straight line which crosses the y axis at p units above(0,0).

I have drawn the data of Fig. 1 in this way, and the result is given in Fig. 2. The fact that the curve in Fig. 2 is a straight line is direct proof of the experimental validity of equation (3) and of its derivative, equation (5). The data of the other six series of experiments show precisely the same thing. For further emphasis Fig. 3 is presented, which gives the data of two other experiments. The three

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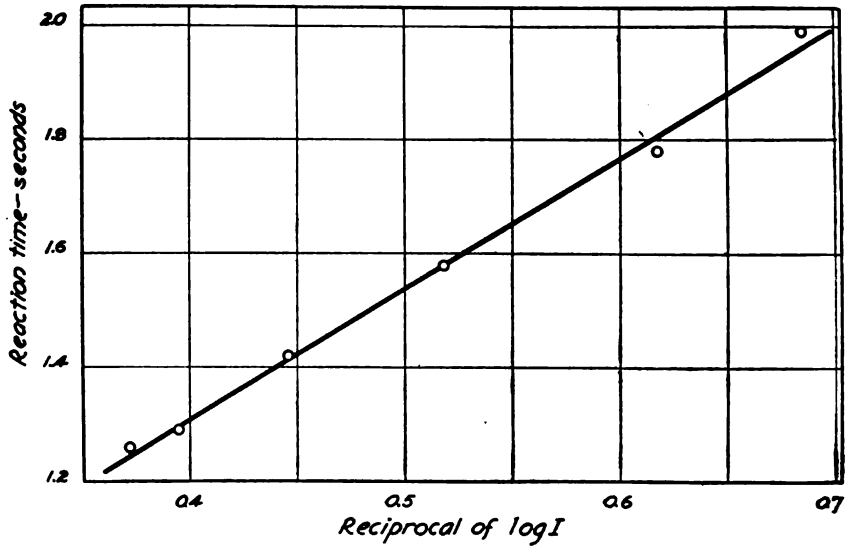

FIG. 2. Relation between the reaction time and the reciprocal of the logarithm of the intensity. Same experiment as Fig. 1.

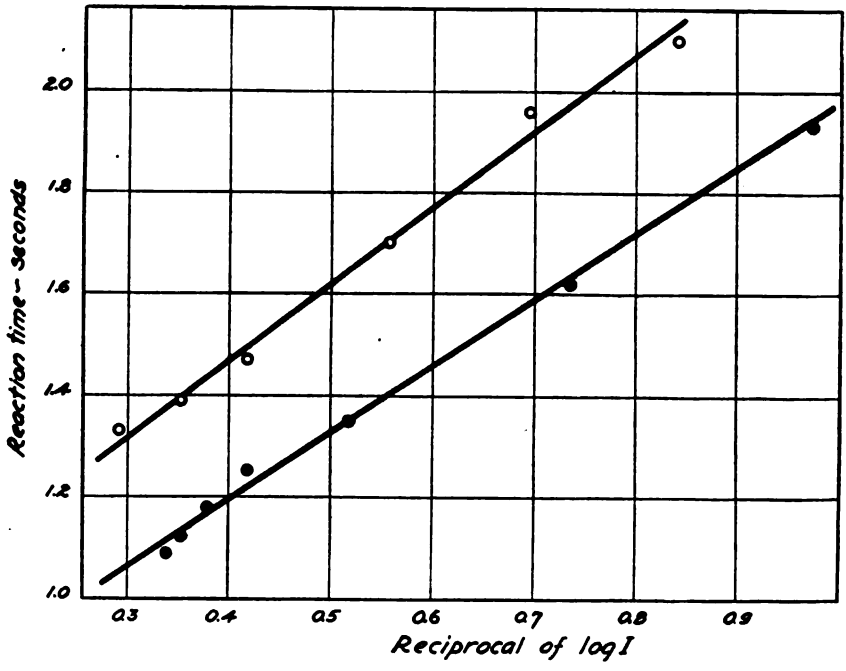

FIG. 3. Relation between the reaction time and the reciprocal of the logarithm of the intensity. Open circles are the data of Experiment 9.9; 250 watt lamp; seven animals; one measurement for each animal for every intensity. Dark circles are the data of Experiment 10.10; 100 watt lamp; seven animals; three readings for each animal for every intensity.

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groups of experiments shown in the two figures were made at intervals of about 3 weeks, with different sources of light, and, of course, with different animals.

There can therefore be no doubt that equations (3) and (5) are true experimentally. The photochemical effect of light in the photosensory stimulation of Mya is then proportional to the product of the logarithm of the intensity of illumination into the time during which the light acts. This result supports previous investigations with *Mya* (*cf.* especially Hecht, 1919–20, *c*), and strengthens the hypothesis proposed to account for its photosensory responses.

SUMMARY.

In its photosensory effect, the action of light depends on two variables,—intensity and time. If the intensity alone is varied, the photochemical effect is proportional to the logarithm of the intensity. If the time alone is varied, the effect is proportional to the time. Experiments here reported show that when both the intensity and the time are varied, the photochemical effect is equal to the product of their separate activities: $E = kt \log I$. These results furnish the means of expressing directly the relation between the intensity of illumination and the reaction time of *Mya*.

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THE RELATION BETWEEN THE WAVE-LENGTH OF LIGHT AND ITS EFFECT ON THE PHOTOSENSORY PROCESS.*

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(Received for publication, November 11, 1920.)

I.

1. In a series of publications I have described several phases of the mechanism which underlies the sensitivity of certain animals (*Mya* and *Ciona*) to light (Hecht, *a*, *b*, *c*, *d*). The basis of this mechanism is essentially a process involving two intimately connected chemical reactions. The first of these is a reversible photochemical reaction

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A$$

in which S is the sensitive substance, P the principal product of decomposition, and A its accessory. The second reaction is an ordinary chemical reaction

$$L \rightarrow T$$

which is catalyzed by the products of reaction of the photochemical reaction. The thermolabile material T is the end-product of the sensory process, and a definite quantity of it is required for the initiation of an impulse which results in the response of the animal to illumination.

The properties of these different substances and reactions are derived from quantitative studies of the responses of animals under conditions of experimentation calculated to bring them out. As the characteristics of the reactions and their components become known

* These experiments were carried out with the aid of a grant from the Elizabeth Thompson Science Fund, to the trustees of which I wish here to express my thanks.

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in greater detail, their identification becomes increasingly possible. Heretofore attention has been centered mainly on the kinetic and dynamic relations of the sensitive system. It is the purpose of the present observations to furnish the basis for the description of a physical property of the photosensitive substance *S*.

2. It is axiomatic to say that in a photochemical transformation only the light which is absorbed is active in producing chemical change. Although the converse,—that all the light which is absorbed gives rise to chemical action,—is not proved, it is well known that the absorption spectrum of a chemical system is intimately connected with its photochemical behavior (Sheppard, 1911, p. 140). Thus, if in a given photochemical system we know the relative effectiveness of a series of lights of different wave-length, it is possible to draw certain conclusions with regard to the absorption spectrum of the sensitive substance in the range of wave-lengths which have been investigated (Lasareff, 1907). Experiments were therefore undertaken to determine the effectiveness of light of different wave-length on the photosensory responses of *Mya arenaria*.

3. The method used is in its essentials as follows. The reaction time of *Mya* to light varies inversely as the logarithm of the intensity (Hecht, 1920–21, *e*). The relation between these two variables may be found for light of any quality. When using monochromatic illumination, the curve expressing the relation between the intensity (*I*) and the reaction time (*t*) may be called an *It-isochrome*, in analogy to the curve at constant temperature called an *It-isotherm*. The experiments consist in mapping out the *It-isochromes* for the photic response of *Mya* to lights of different composition. The relations among the isochromes will give the relative effectiveness of the different lights.

The experiments were performed in the Marine Biological Laboratory at Woods Hole, Massachusetts, during the summer of 1920.

II.

1. Monochromatic light is secured by means of Wratten Light Filters Nos. 70 to 76 inclusive. These are made by the Eastman Kodak Company, and consist of specially stained gelatin films cemented between glass plates. The absorption spectra of these light filters

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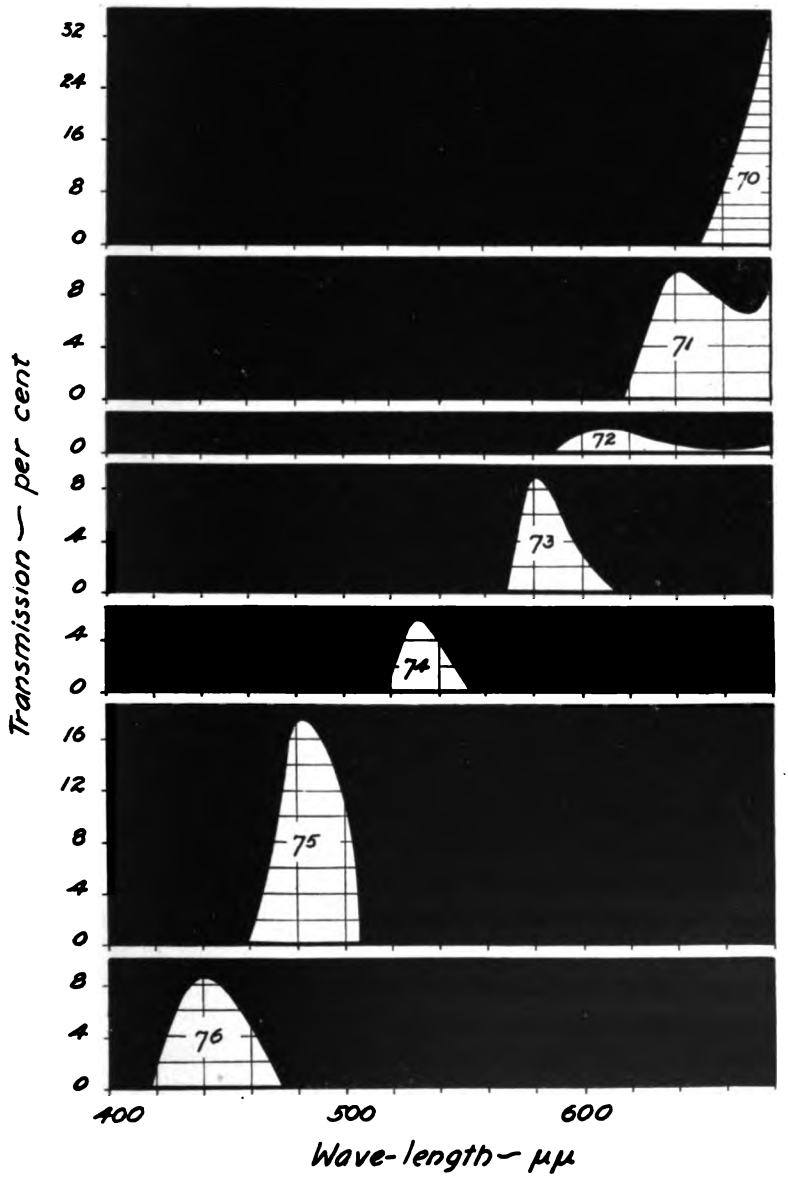

FIG. 1. Transmission spectra of the Wratten Light Filters used to secure monochromatic illumination.

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have been carefully measured.[1] They are presented graphically in Fig. 1. The white spaces are the transmission areas for the different screens in terms of the per cent transmission of the incident light.

It is apparent that each screen transmits a rather narrow portion of the visible spectrum. In the significant part of the spectrum (λ = 400 to 600 $\mu\mu$) the screens transmit bands about 40 $\mu\mu$ wide; most of the light transmitted is confined to an even narrower band.

2. With these screens I used a 400 watt, concentrated-filament Mazda lamp, running on an ordinary lighting circuit of 115 volts. This source of light may be considered as a point source, and different relative intensities may be obtained by placing animals at different distances from the light, the intensities being computed on the inverse square law.

For ordinary sources of light the energy content of the spectrum varies with the wave-length. It is therefore important to know the exact values of the relative energy distribution of the light in order that the energy transmitted by the filters be known. The results of the determination of the energy distribution for the 400 watt lamp which I used in these experiments are shown in Fig. 2, which gives the relative energy content of the different wave-lengths of the visible spectrum.

From Fig. 2 we know the energy incident on the filters. Fig. 1 in turn tells us the portion of this incident energy which is transmitted by each of the filters. It is thus a simple computation to find out the energy content of the light transmitted by each filter in conjunction with the lamp. The transmission values in Fig. 1 and the energy values in Fig. 2 are both determined for bands 10 $\mu\mu$ wide. The total energy transmitted by any filter is therefore the sum of the energy transmitted by its constituent 10 $\mu\mu$ bands. These totals are given in Table I.

The Mazda lamp plus a screen may thus be considered as a source of energy which radiates a narrowly defined portion of the spectrum, and whose intensity is proportional to the energy content of the transmitted light. For convenience I have used for the relative intensity the same figures as those given in Table I for the transmitted energy.

[1] The measurements are given on p. 61 of a booklet—Wratten Light Filters—published by The Eastman Kodak Company.

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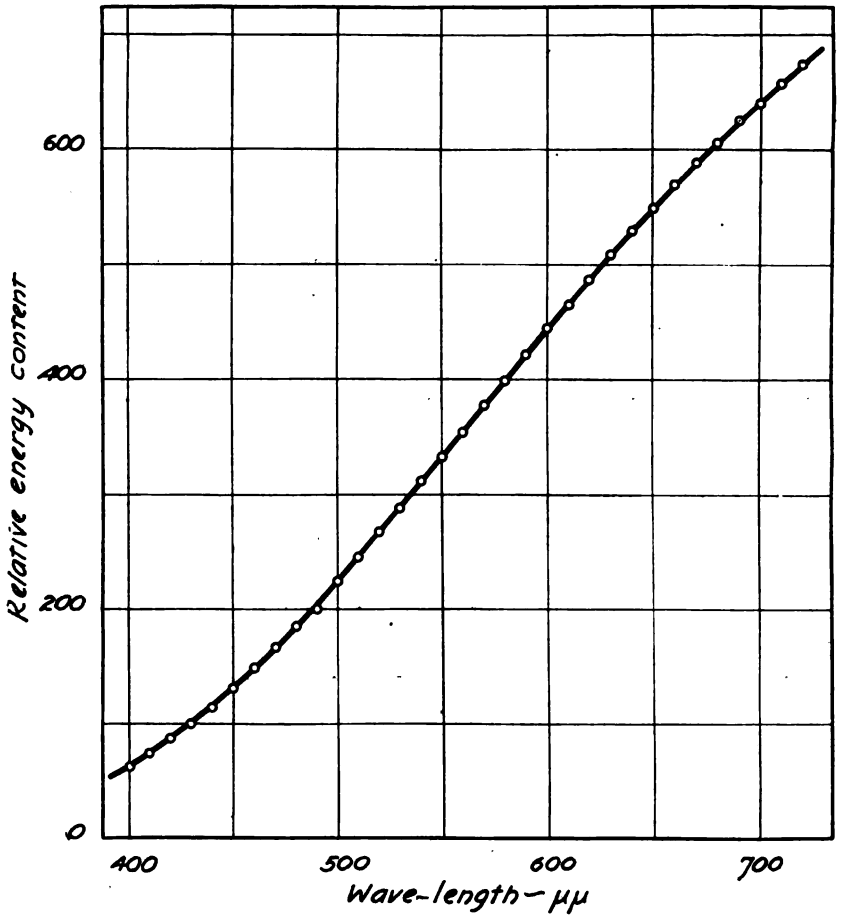

FIG. 2. Energy distribution in the spectrum of the 400 watt lamp used in conjunction with the light filters.

TABLE I.

Relative Energy Transmitted by Wratten Light Filters Plus a 400 Watt Lamp.

Filter No.	Energy.
70	366.8
71	285.3
72	34.8
73	88.6
74	36.8
75	106.9
76	38.1

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The distribution of the energy transmitted by each screen is given in Fig. 3. For uniformity the total transmission is given the value of 100. It will be seen that the point of maximum energy transmission is occasionally shifted toward the red end as compared with the per cent transmission given in Fig. 1. This is, of course, because the energy content of the spectrum increases steadily toward the red end, as shown in Fig. 2.

3. In Figs. 1 and 3 the transmission of the filters is given only for the visible spectrum. All the filters transmit infra-red rays. It may, however, be said at once that rays beyond $\lambda = 680\ \mu\mu$ are ineffective as stimulating agents for *Mya*. I have determined this by subjecting animals to very intense illumination from which all the visible rays have been filtered out by means of Wratten Light Filter No. 87. This screen transmits the rays beyond $\lambda = 680\ \mu\mu$, and none below that wave-length. In no case did the animals respond to these infra-red rays. As a source of error, therefore, infra-red rays may be left out of consideration.

The screens do not transmit ultra-violet light. In addition, the light from the incandescent filament has to pass through several centimeters of glass before reaching the animal. Therefore, the ultra-violet rays are screened out in this way as well, and do not enter as a source of error.

The filters are quite photostable. But in order to avoid any possible bleaching effect, a shutter is placed between the light source and the filter, so that the filter is exposed to light only during the few seconds required to expose an animal and to measure its reaction time. Moreover, the light is turned off between exposures in order that as little heat be formed as possible. This is a necessary precaution, because a 400 watt lamp produces a tremendous amount of heat, which might otherwise pass even through the shutter and affect the gelatin between the glass plates of the filter.

From what has been said it is evident that these Wratten Light Filters furnish a simple method of procuring practically monochromatic light of known intensities, many times greater than can ordinarily be produced with the aid of a dispersion spectrum.

4. Further than the use of the screens, the experiments involve no new principle. A filter is placed in the path of the beam of light im-

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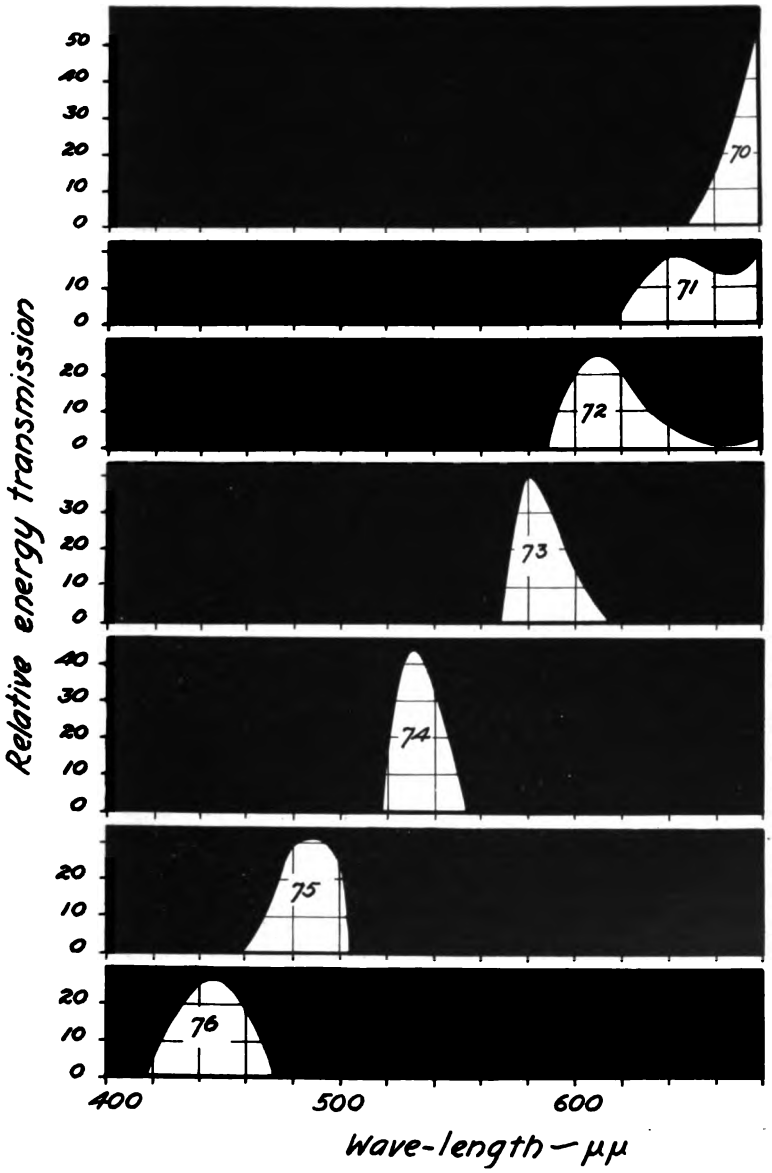

FIG. 3. Energy distribution of the light transmitted by the filters when used with the 400 watt lamp. The total energy for each filter is placed arbitrarily at 100. *Cf.* Table I.

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mediately in contact with the shutter (for a description of the apparatus used, see Hecht, 1919–20, *c*). Several animals which have been thoroughly dark-adapted are then used. The reaction time of each animal is measured with a stop-watch. After a rest of 15 minutes in the dark, the animals are again tested, but at a distance from the light nearer or farther than before, depending on the magnitude of the previous reaction time. After another period in the dark, the reaction time is measured at still another intensity, and the process repeated until enough responses have been measured to cover a range between 2.0 and 4.0 seconds in the reaction time.

The curve giving the relation between the reaction time and the intensity is the *It*-isochrome for this filter. The entire procedure is then repeated with another filter, until all the filters have been tested. In this way a series of *It*-isochromes are mapped out for the seven portions of the visible spectrum shown in Fig. 3.

III.

1. Several preliminary experiments agreed in showing that the light coming through Filter 75 is the most effective portion of the spectrum. A final set of experiments was then arranged using nine dark-adapted animals. The results are given in Fig. 4. Each point is the average of nine determinations of the reaction time, one for each animal. The curves are the *It*-isochromes drawn smoothly through the points. An exception is the curve for Filter 76, which for obvious reasons is drawn parallel to the other six curves.

The range of intensities required to produce similar responses is very large; from 2 to 16,000 units. To present them in a single figure is manifestly impractical. Moreover, it has been shown that the reaction time is inversely proportional to the *logarithm* of the intensity. Consequently, the abscissæ of Fig. 4 are the logarithms of the intensities and not the intensities themselves. The use of such a logarithmic plot makes all the *It*-curves parallel to one another, and renders their comparison more simple than would otherwise be the case.

The *It*-isochromes of Fig. 4 show at once that the most effective portion of the spectrum is the light transmitted by Filter 75. This corresponds to a band whose maximum is at 490 $\mu\mu$, in the blue-green.

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It requires the least amount of energy to produce a given photosensory effect.

2. It will be of significance to determine quantitatively the relative effectiveness of the different parts of the spectrum. The method used in this connection deserves a little consideration. It is required to find the relative effectiveness of a series of reagents on a given system. The common procedure of comparing the different effects produced by the same concentration of the reagents, though giving qualitative results, is utterly fallacious for quantitative purposes, except in rare instances such as when the effect produced is a linear function of the

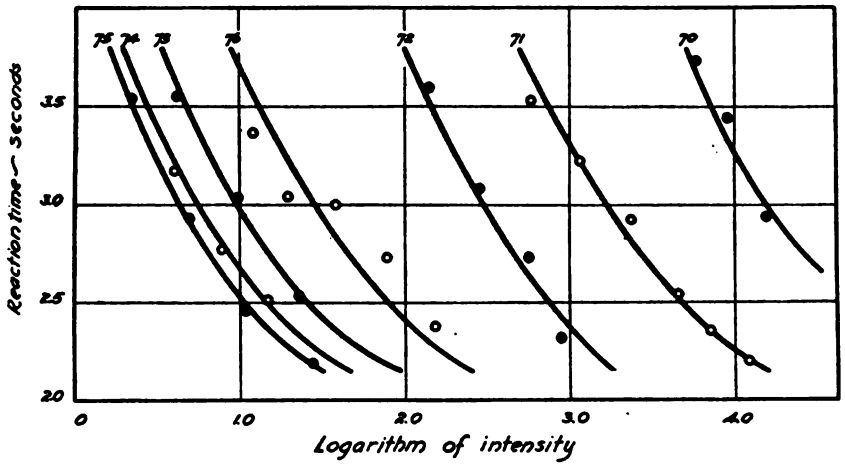

FIG. 4. *It*-isochromes for the responses of *Mya* to the different lights transmitted by the various filters.

concentration. A quantitatively correct evaluation requires the comparison of the concentrations of the different reagents which will produce the same effect.

I need not labor the point, because Brooks (1920) has presented it in some detail in its bearing on the theoretical interpretation of hemolysis data. Applied to our immediate problem, it follows that the important thing in working with spectral light is *not* to have a spectrum of *equal* energy distribution with which to determine the relative effects of different parts of it, but to have a spectrum of variable, *known* energy distribution, with which to determine the

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energy required to produce the *same* sensory effect. The value of the latter method will become apparent when the attempt is made in the next section to interpret the experimental findings.

In the present instance the same sensory effect is represented by responses produced in the same reaction time. It is therefore required to measure the different intensities necessary to produce the same reaction time. This can be done by reading from the *It*-isochromes of Fig. 4 the intensities corresponding to definite values of the reaction time.

I have done this for three values; for 2.5, 3.0, and 3.5 seconds. In each instance the intensity for Filter 75 is placed at unity, and the relative intensities of the others computed in terms of it. The three values so obtained for each portion of the spectrum are given in Table II. In treating them further, I have used the average of the three figures for each filter.

TABLE II.

Relative Intensities Required to Produce the Same Reaction Time.

Reaction time.	Intensity for filter No.						
	70	71	72	73	74	75	76
sec.							
2.5	4,030	452	66.9	22.8	13.2	1.0	5.1
3.0	3,650	392	65.4	22.0	12.9	1.0	6.5
3.5	3,240	312	63.0	20.7	12.6	1.0	5.3
Average...	3,640	385	65.1	21.8	12.9	1.0	5.6

3. The relative effectiveness of the different lights may be represented by the reciprocals of the relative intensities. Fig. 5 shows these values in graphic form. For convenience, and for another reason which will presently become apparent, the reciprocal of the maximum is made equal to 100, the other values being computed accordingly and employed as ordinates. The wave-length is used as abscissa. Each point has been plotted as a rectangle, whose center is the point of maximum energy transmission as given in Fig. 3, and whose horizontal dimension represents the points between which 75 per cent of the transmitted energy is confined. A smoothed curve through the points shows strikingly that the most effective portion

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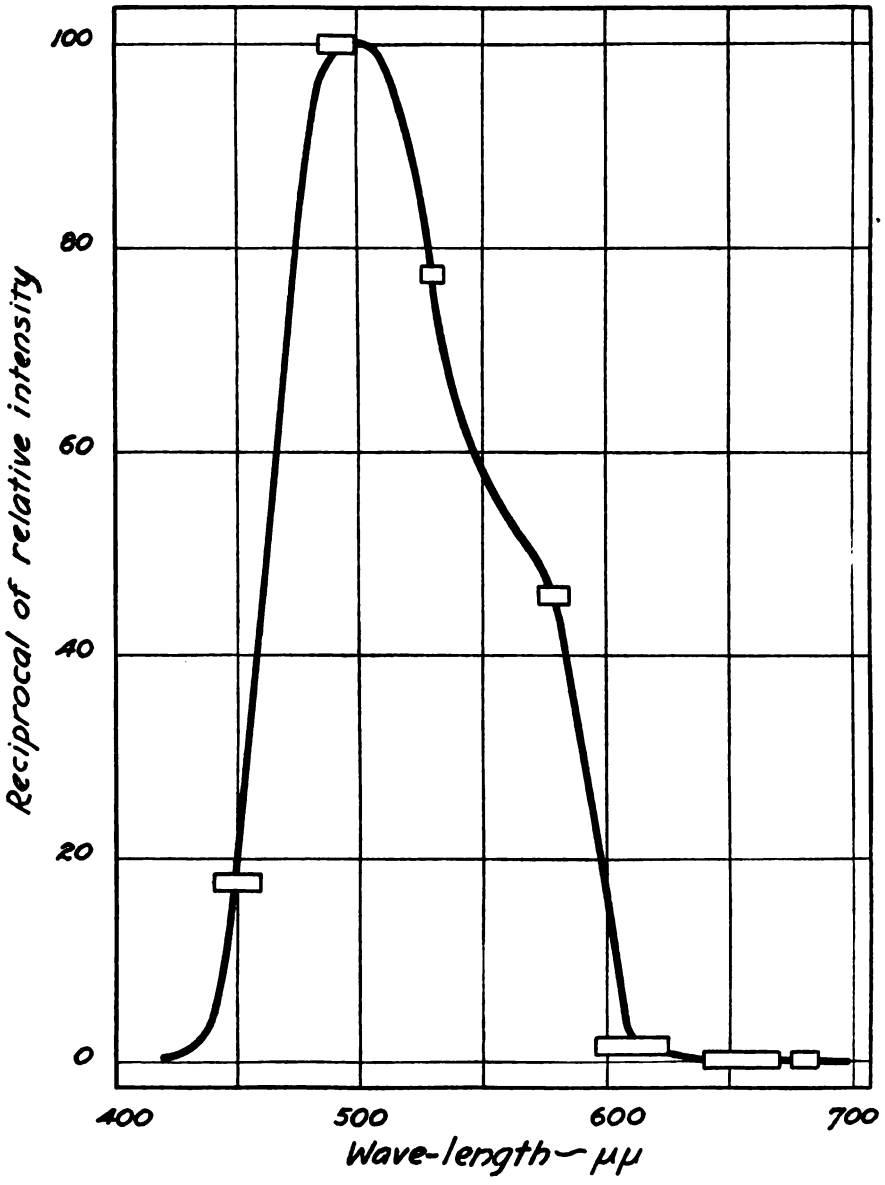

FIG. 5. Relative effectiveness of the spectrum in sensory stimulation. The curve also represents the absorption spectrum of the photosensitive substance of *Mya*. In the latter case the ordinates represent per cent of absorption by the sensitive substance.

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of the spectrum for the sensory stimulation of *Mya* lies between 490 and 510 $\mu\mu$. The effectiveness of the spectrum drops rapidly on both sides of this maximum, the parts above 610 $\mu\mu$ and below 420 $\mu\mu$ being practically ineffective as stimulating agents.

IV.

1. On the basis of certain considerations it is possible to place a physical interpretation on the results as presented in Fig. 5 and Table II. Lasareff (1907) has shown that for dyes that are bleached rapidly by light, the photochemical effect is directly proportional to the energy absorbed per interval $\Delta\lambda$, independent of the value of λ and of the position of the absorption maximum. These results have been confirmed on other photochemical reactions (Weigert, 1911, p. 90); and a similar state of affairs has been shown to be true for the bleaching of visual purple (Trendelenburg, 1911).

This means that in order to produce a given photochemical effect, sufficient light of any wave-length λ_n must be delivered, such that the amount absorbed will be the same at all values of λ_n. The relative effectiveness of different parts of the spectrum therefore depends solely on the absorption spectrum of the sensitive substance. The greater the proportion of light of a given wave-length that is absorbed, the less of that light is necessary to produce a given effect.

2. If we apply these findings to the present data, it will become apparent that Fig. 5 represents the absorption spectrum of the photosensitive substance S in the reaction

$$S \rightarrow P + A$$

which is responsible for the photic sensitivity of *Mya*.

Let us assume that in the position of maximum effectiveness (λ_{max} = 500 $\mu\mu$) there is complete absorption of the incident energy. At any other value of λ_n the same amount of energy will be required to be absorbed in order to produce the same effect. But the amount of incident energy is greater than this, in inverse proportion to the magnitude of absorption at that wave-length. We know the incident energy from Figs. 4 and 5 and Table II. The absorbed energy is equal to the amount required at the maximum effectiveness (λ =

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500 $\mu\mu$). At any other wave-length λ_n, then, the per cent of absorption will be given by the ratio of the energy at λ_{max} divided by the incident energy at λ_n.

The absorption at λ_{max} will then be 100 per cent, and at any other value of λ_n it will be equal to the reciprocal of the incident energy. Therefore Fig. 5 represents the absorption spectrum of the photosensitive substance *S*, the ordinates now being per cent of absorption. Absorption spectra are not infrequently given in this way (Henri, 1919, p. 42, 65). I shall therefore not give the absorption spectrum in terms of the absorption index. The latter can be calculated according to certain assumptions as to the thickness of the absorbing layer etc. (*Cf.* Bovie, 1918–19), in themselves, however, only of speculative interest.

3. Because of its interpretation as the absorption spectrum of *S*, the shape of the curve in Fig. 5 becomes of significance. Many known substances possess absorption bands in position and extent similar to that shown here (*Cf.* Uhler and Wood, 1907, Fig. 17 and those following). As a rule such curves are symmetrical with regard to the point of maximum absorption. This, however, is by no means universal, because many substances show skew absorption spectra similar to that in Fig. 5; for example, uranine, as studied by Uhler and Wood (1907, Fig. 15). In such cases it is usual to assume that there are really two, or more, vibrators in the molecule, their combined effect being given by the total curve as found. If we suppose that in the photosensitive substance *S* there are present two vibrators, one whose period corresponds to 500 $\mu\mu$ and the other to about 570 $\mu\mu$, each giving a symmetrical resonance curve, the compound curve of Fig. 5 would be their resultant.

Leaving aside these speculative matters, it may be noted that the appearance of the absorption curve, though not particularly distinctive, is sufficiently so to serve as a corroboration of the identity of the photosensitive substance *S* in future experimentation.

V.

In recent years there has appeared a number of careful measurements of the most effective portion of the spectrum for the stimulation of different organisms (Laurens and Hooker, 1920; Loeb and

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Wasteneys, 1916; Mast, 1917). Although for many animals this point lies in the yellow-green, and for many plants in the blue-green, there is an increasing number of organisms which do not conform to this classification. *Mya arenaria* clearly belongs in this non-conforming category, because it is an animal for which the maximum effectiveness is in the blue-green.

The particular significance of the above attempts at classification is due to Hess (1910). As the result of experiments with a number of animals, Hess has come to the conclusion that "the curves of the relative stimulating values of different homogeneous lights coincide, approximately or exactly, with the luminosity curve of the totally color blind human eye" (Hess, 1910, p. 362). From which he proceeds to draw certain conclusions with regard to the nature of the responses of animals to light. Loeb and Wasteneys (1916) have shown that these conclusions not only fail to agree with the facts of other investigators, but also that the process of arriving at them involves a decided logical fallacy. If after this any additional evidence were needed, the results with *Mya* certainly show that Hess' original statement is not generally true. The curve of effectiveness of the spectrum for *Mya* possesses nothing in common with the luminosity curve for the color blind or the dark-adapted human eye.

It may be added that Hess' method of experimentation is open to criticisms which invalidate his conclusions. Working with certain clams whose photic sensitivity is similar to that of *Mya*, he employs criteria of the effectiveness of light which cannot be considered quantitative. He notes the length of the extended siphon, or the extent of its retraction as the animal is moved along the projected spectrum. My experience with *Mya* has been that these are variable characteristics, even when care is taken to insure uniformity otherwise. In Hess' experiments no values are given for the energy content of the spectrum; no time is allowed for the recovery of an animal in the dark between stimulations; no measurements are made of the time of exposure; no record is made of the reaction time. In fact, few measurements of any kind are given. Even the "curves" on which the comparison with the human eye rests fail to appear in the article (Hess, 1910). Thus, even if Hess' logic were correct, his experimental findings are open to grave doubt; and the conclusions drawn from them are therefore doubly invalid.

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The results with spectral lights therefore sum up to this. With *Mya* the effectiveness of the spectrum depends exclusively on the absorption spectrum of the photosensitive substance in the sense organ. The different positions of the maximum effectiveness for various organisms merely show that the sensitive substances concerned are different entities, each possessing its own absorption spectrum.

SUMMARY.

1. Following the description of a simple method of securing high intensities of monochromatic illumination, it is shown that the most effective portion of the spectrum for the stimulation of *Mya* is near $\lambda = 500\ \mu\mu$.

2. The quantitative data secured is interpreted in terms of certain photochemical findings, and as a result the absorption spectrum of the photosensitive substance of *Mya* is tentatively mapped out.

Through the courtesy of Doctor M. Luckiesch of the Nela Research Laboratory, Mr. W. E. Forsythe and Mr. F. E. Cady determined the energy distribution for the 400 watt lamp which I used in these experiments. I take pleasure in thanking them for their help in this connection.

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ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS.

III. The Action of Salts in Low Concentration.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 9, 1920.)

I. The Difference in the Effect of Acids, Alkalies, and Salts on Proteins.

The data concerning electrolytic dissociation and the behavior of electrolytes in general suggest that it is well to discriminate between solutions of electrolytes in low and in high concentrations. While no sharp line of demarcation can be drawn it will suffice for the problem we are discussing to designate a concentration of electrolytes below M/16 as low and those near the solubility limit of one of the components as high. In this paper we intend to deal with solutions of low concentrations; *i.e.*, M/16 or less.

It has been noticed by a number of authors that the influence of neutral salts on the physical properties of proteins differs from that of acids and bases; and various attempts have been made to find an expression for this difference. Pauli[1] states that while acids and alkalies form salts with proteins, neutral salts form "adsorption compounds" with "electrically neutral," *i.e.* non-ionized, protein molecules, both ions of the salt being simultaneously adsorbed by the protein molecule. This idea is no longer tenable for salt solutions of low concentration since the writer has shown through his experiments with powdered gelatin that only one (or practically only one) of the two ions of a neutral salt can combine at one time with a protein. At the isoelectric point, *i.e.* at pH 4.7, gelatin can combine with neither ion of a neutral salt; at a pH > 4.7 only the metal ion of the neutral salt can combine with the gelatin, forming metal gelatinate;

[1] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

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at a pH < 4.7 only the anion of the neutral salt is capable of combining with the protein, forming gelatin-acid salts.[2]

Lillie has made the statement that while acids and alkalies increase, salts depress the osmotic pressure of gelatin.[3] This statement, while it was the expression of facts actually observed by Lillie, is no longer tenable owing to the fact that the influence of the hydrogen ion concentration of the gelatin solution was not taken into consideration. If we add acid to a gelatin-acid solution of a pH of 3.0 or below, the effect is practically the same as when we add a neutral salt, namely a diminution of the osmotic pressure of the solution; and when we add alkali, *e.g.* KOH, to a solution of a metal gelatinate of pH 11.0 or above, the effect is also a similar depression of the osmotic pressure as that caused by the addition of KCl. We also get a depression when we add some acid to a solution of metal gelatinate or when we add some alkali to gelatin-acid salts; since in both cases the gelatin is brought nearer to the isoelectric point.

It is also incorrect to speak of an antagonism between the effects of acids and salts, since the facts mentioned show that there is also an antagonism between little and much acid; thus if the pH of a gelatin-acid salt is 3.0 a further addition of acid depresses the osmotic pressure or viscosity. The question then arises, what is the correct expression of the facts in the case?

An analogy with another field of phenomena may be of service. The writer has recently published a series of articles on the influence of electrolytes on the rate of diffusion of water through collodion membranes, which have shown that water diffuses through such a membrane as if the particles of water were positively charged. When pure water is separated from a solution of an electrolyte of not too high a concentration, the positively charged particles of water diffuse through the collodion membrane into the solution as if they were attracted by the anion and repelled by the cation with a force increasing with the valency of the ion. In this case the oppositely charged ions of an electrolyte influence the rate of diffusion of water through the membrane in an opposite sense.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237; *Science*, 1920, lii, 449.
[3] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

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The second fact which was brought out was that the relative strength of the apparent attractive and repelling action of the oppositely charged ions of an electrolyte upon the electrically charged particles of water varies with the concentration of the electrolyte in the solution. In the lower concentrations of the electrolyte the attractive action of the anion upon the positively charged particles of water increases more rapidly with increasing concentration of the salt than the repelling action of the cation; while when a certain concentration is reached the repelling action of the cation upon the positively charged particles of water increases more rapidly with a further increase in the concentration of the salt than the action of the anion.[4]

These facts offer an analogy which is helpful in understanding the difference in the action of acids and alkalies on the one hand, and of neutral salts on the other upon the physical properties of proteins.

When acids or alkalies are added to isoelectric gelatin both ions of the acid or alkali influence the physical properties of proteins but in an opposite direction. When we add acid to isoelectric protein the hydrogen ions increase, the anions depress the osmotic pressure and viscosity of the protein solution (and this depressing action increases with the valency of the anion of the acid). As long as little acid is added to isoelectric protein the augmenting action of the hydrogen ion on these properties increases more rapidly with increasing concentration of the acid than the depressing action of the anion; while when the pH of the solution falls below 3.3 or 3.0 the reverse is the case. This causes the drop in the curves for osmotic pressure, viscosity, and swelling below a pH of 3.0.

When we add alkali to isoelectric protein the OH ions (or the diminution of the concentration of hydrogen ions) increase the osmotic pressure, viscosity, etc., of the solution of metal proteinate while the cation of the alkali depresses these properties with a force increasing with the valency of the cation. In the lowest concentrations of the alkali added the augmenting action of the OH ion on the physical properties of the metal proteinate increases more rapidly with the concentration than the depressing effects of the cation of the alkali; while in higher concentrations, *i.e.* as soon as the pH becomes about 10.0 or 11.0, the reverse is the case.[5]

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247.

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When, however, neutral salts are added to protein solutions we no longer notice an opposite effect of the oppositely charged ions. When neutral salts are added to isoelectric gelatin no effect is noticed as long as the concentration of salt does not reach the value required for precipitation. When neutral salt is added to a protein solution on either side of its isoelectric point only a depressing action of that ion which has the opposite sign of charge as the protein ion is observed. No augmenting action of the ion with the same sign of charge as the protein is noticeable. Thus if we add $CaCl_2$ or Na_2SO_4 to a solution of gelatin chloride or gelatin nitrate we observe only a depressing effect of the Cl or SO_4 ion but no augmenting effect of the Ca or Na ion; while when we add these salts to a solution of a metal gelatinate we observe only a depressing effect of the Ca or Na ion but no augmenting effect of the anion. We shall first show that this is the correct expression for the difference in the action of acids and bases on the viscosity of proteins on the one hand and of neutral salts on the other.

A 2 per cent solution of isoelectric gelatin is prepared and brought to a pH of 4.0. The solution is made 1 per cent in regard to the originally isoelectric gelatin by adding to 50 cc. of the 2 per cent solution either 50 cc. of H_2O or of a salt solution, *e.g.* NaCl, of different molecular concentration, from M/8,192 to 1 M, taking care that the hydrogen ion concentration remains the same. We determine the viscosity (*i.e.* the time of outflow through a viscometer) in the way described in a preceding publication and plot the reciprocal of time of outflow (counting that of water as 1) as ordinates over the pH as abscissæ (lower curve, Fig. 1). For the sake of briefness we beg leave to designate this value as specific viscosity.[5] The addition of the NaCl causes only a drop, and no rise in the curve.

If, however, we mix the 2 per cent gelatin solution of pH 4.0 with various concentrations of HCl (upper curve, Fig. 1) instead of with NaCl we do not notice a drop but at first a rise followed by a drop when the concentration of the Cl ion is a little above N/1,000. In Fig. 1 the drop appears at a concentration of about N/256 HCl, but the reader must remember that on account of the fact that part of the acid combined with the gelatin the pH of the solution was about 3.0. In other words, while the addition of H ions increases the vis-

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cosity of a solution of gelatin chloride of pH 4.0, the addition of Na ion does not have such an effect, but the Cl ion depresses the viscosity in both cases, no matter whether NaCl or HCl is added to the gelatin

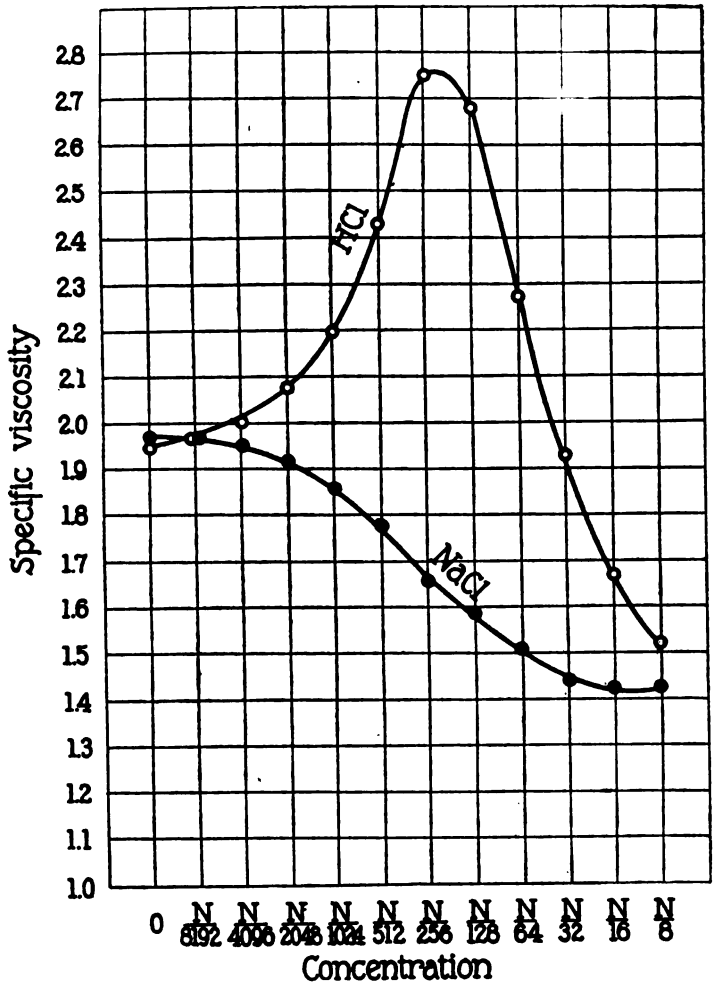

FIG. 1. Difference in the effect of different concentrations of NaCl and of HCl on the specific viscosity of a 1 per cent solution of gelatin chloride of pH 4.0. In the case of NaCl we observe only the depressing effect of the Cl ion; in the case of HCl we notice an augmenting effect of the H ion and a depressing effect of the Cl ion, the latter prevailing as soon as the concentration of acid added is > N/256.

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solution; and the depressing action of the Cl ion increases with its concentration. Moreover, the increase of the viscosity by the H ions stops as soon as the pH of the solution reaches about 3.0.

When we repeat the same experiment with a gelatin solution of pH 3.0, the addition of NaCl immediately causes a drop also (Fig. 2)

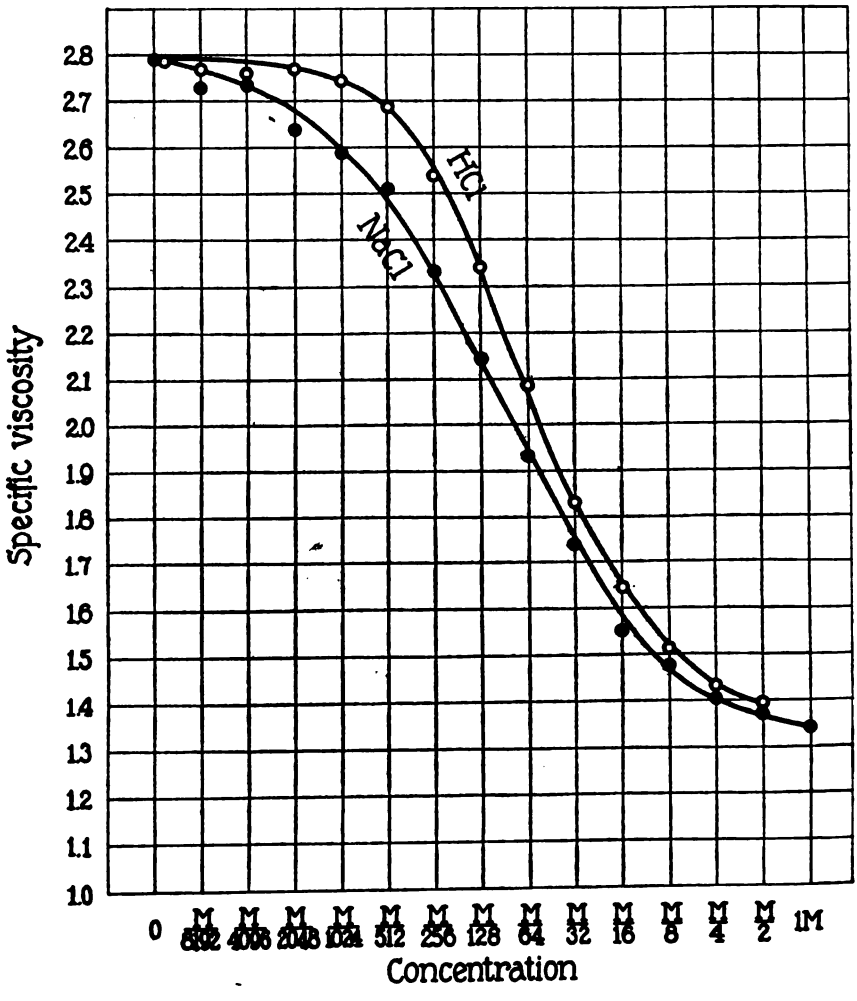

FIG. 2. The specific viscosity of 1 per cent solution of gelatin chloride of pH 3.0 is depressed almost equally by the Cl ion of HCl as of NaCl. The augmenting effect of the H ion in the case of HCl is no longer noticeable.

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while the addition of HCl no longer causes a rise but the drop commences a little later than in the case of NaCl.

When, however, we make the same experiment with a gelatin solution of pH 2.5 (Fig. 3), we notice an immediate drop upon the

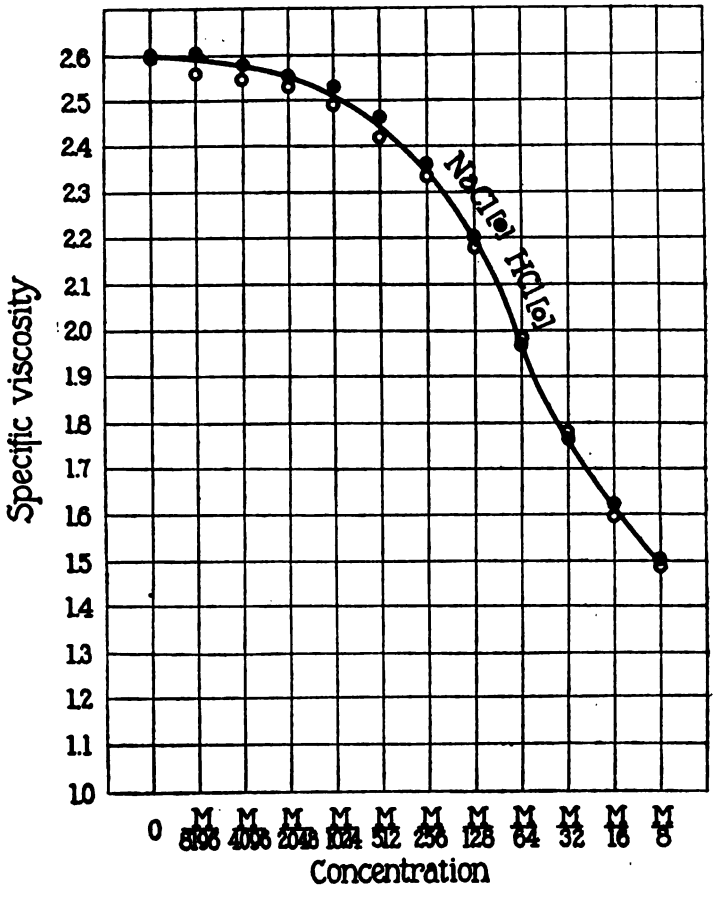

FIG. 3. When the gelatin solution has a pH of 2.5, HCl and NaCl depress the specific viscosity of the gelatin solution to the same degree.

addition of HCl as well as in the case of the addition of NaCl, and the curve for HCl coincides practically with that for NaCl, as our theory demands.

That the depression of the viscosity of gelatin chloride due to the presence of a salt is exclusively determined by the anion of the salt

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and that the cation has no augmenting effect is shown in Fig. 4, where the influence of NaCl, $CaCl_2$, and $LaCl_3$ upon the viscosity of gelatin of pH 3.0 is represented. 50 cc. of a 2 per cent solution of gelatin chloride of pH 3.0 were added to 50 cc.of a solution of different concentrations of each salt as described, the pH being kept at 3.0. It is

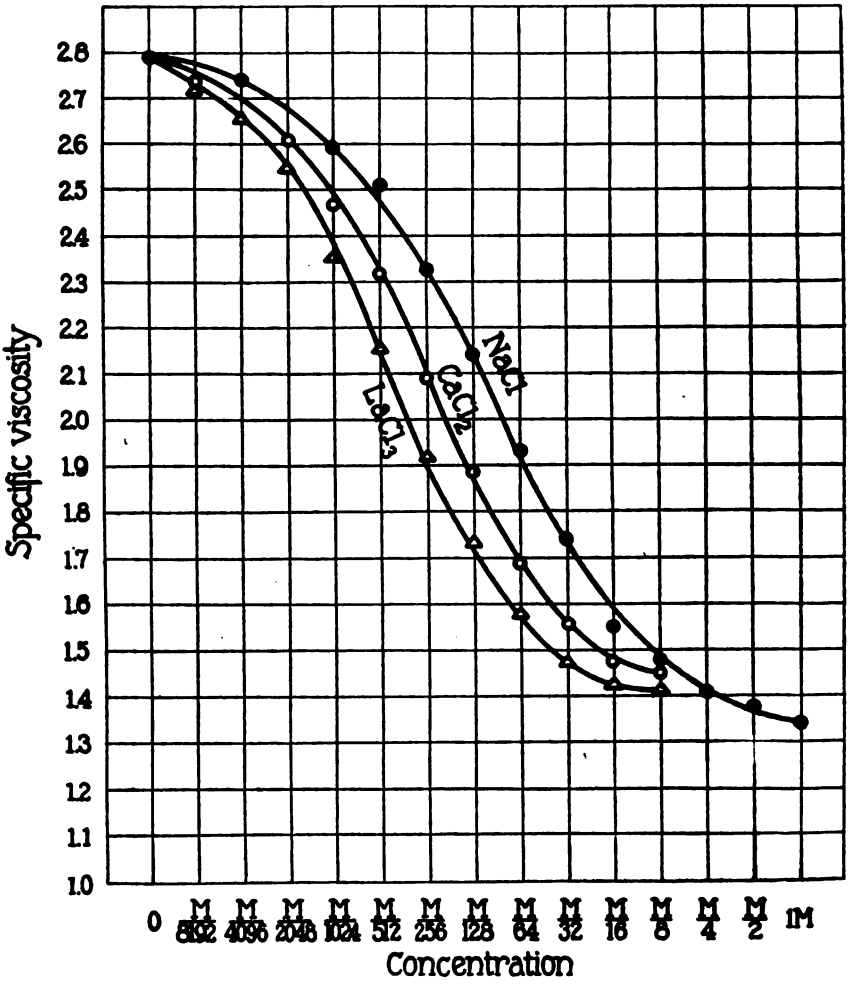

FIG. 4. The depressing effect of equal molecular concentrations of NaCl, $CaCl_2$, and $LaCl_3$ on the specific viscosity of 1 per cent gelatin chloride solution of pH 3.0 is in exact proportion to the concentration of the Cl ions in the solution; *i.e.*, as 1:2:3.

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obvious from Fig. 4 that the molecular concentrations of NaCl, $CaCl_2$, and $LaCl_3$, which depress the viscosity to the same level are approximately in the ratio of 3:2:1. Thus when the effect of NaCl and $CaCl_2$ is plotted over the same concentration of the Cl ions the curves for the salts become identical (Fig. 5), and the same would be practically

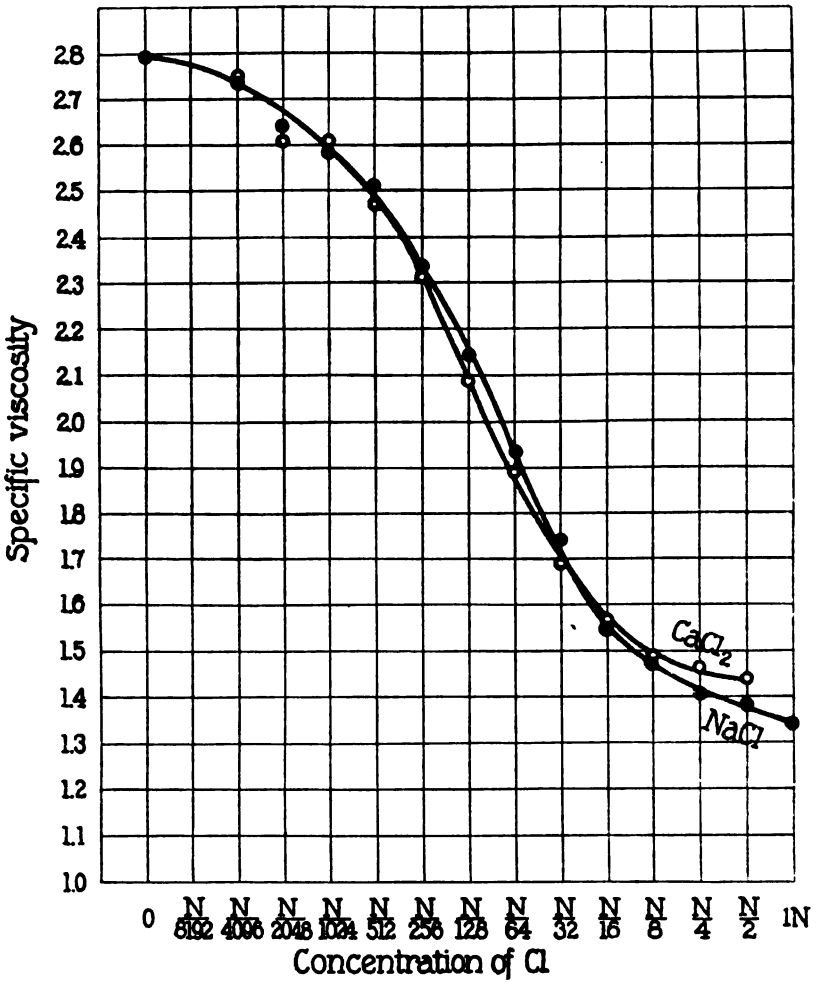

FIG. 5. Showing that NaCl and $CaCl_2$ have the same depressing effect on the viscosity of gelatin chloride of pH = 3.0 when the concentration of Cl ions is the same.

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true for the $LaCl_3$ curve. From this it follows that the depressing effect of these three salts on gelatin chloride is practically exclusively a function of the concentration of the Cl ion, while no effect of the cation seems to be noticeable. In all these cases the pH of the gelatin solution was not altered by the addition of the salt.

When we prepare 1 per cent solutions of gelatin chloride of pH 3.0 in solutions of Na salts with the anion of a weaker acid, *e.g.* Na_2 oxalate, $Na_4Fe(CN)_6$, the pH is increased and we are in danger of attributing erroneously a depressing effect to the anion which in reality is caused by the increase in pH. In Fig. 6 the effects of the addition of equal concentrations of NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ on gelatin chloride of pH = 3.0 are plotted. In the case of $Na_4Fe(CN)_6$ only the lowest concentrations, from M/8,192 to M/1,024, could be used, since in these only did the pH of the protein solution remain = 3.0. Fig. 6 shows that the depressing effect of these salts increases rapidly with the valency of the anion. When the concentration of the salt was only M/1,024 a drop in the viscosity was already noticeable. This drop was small in the case of NaCl (from 2.8 to 2.6), was greater in the case of Na_2SO_4 (from 2.8 to 2.35), and considerably greater in the case of $Na_4Fe(CN)_6$ (from 2.8 to 1.5). The objection might be raised that since Na_2SO_4 has twice as many cations as NaCl of the same concentration and $Na_4Fe(CN)_6$ has four times as many cations, it was the difference in the concentration of the cations which caused the difference in the drop. This is refuted by the fact that Na_2SO_4 causes a drop to 1.8 at a concentration of M/256 while NaCl causes the same drop at a concentration of above M/64 which is about four times as high. If the concentration of the cation were responsible for the drop the two concentrations should be as 1:2. $Na_4Fe(CN)_6$ causes the same drop of the viscosity to 1.8 at a concentration less than M/1,024. Hence the concentration of $Na_4Fe(CN)_6$ required to cause the same diminution of the specific viscosity as that caused by M/64 NaCl is less than $\frac{1}{16}$ of the latter, while it should be at the least only $\frac{1}{4}$ if the cation were responsible for the drop. The depressing effect of the anion seems to increase almost in proportion to the square of its valency, as the Hardy-Whetham rule demands.

We have selected viscosity experiments, but experiments on osmotic pressure and on swelling lead to the same formulation of the difference in the effect of acids and salts.

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What has been shown for the effect of acids on the physical properties of proteins can also be shown for the influence of alkalies. Thus the addition of KOH to Na gelatinate of pH 12.0 depressed the

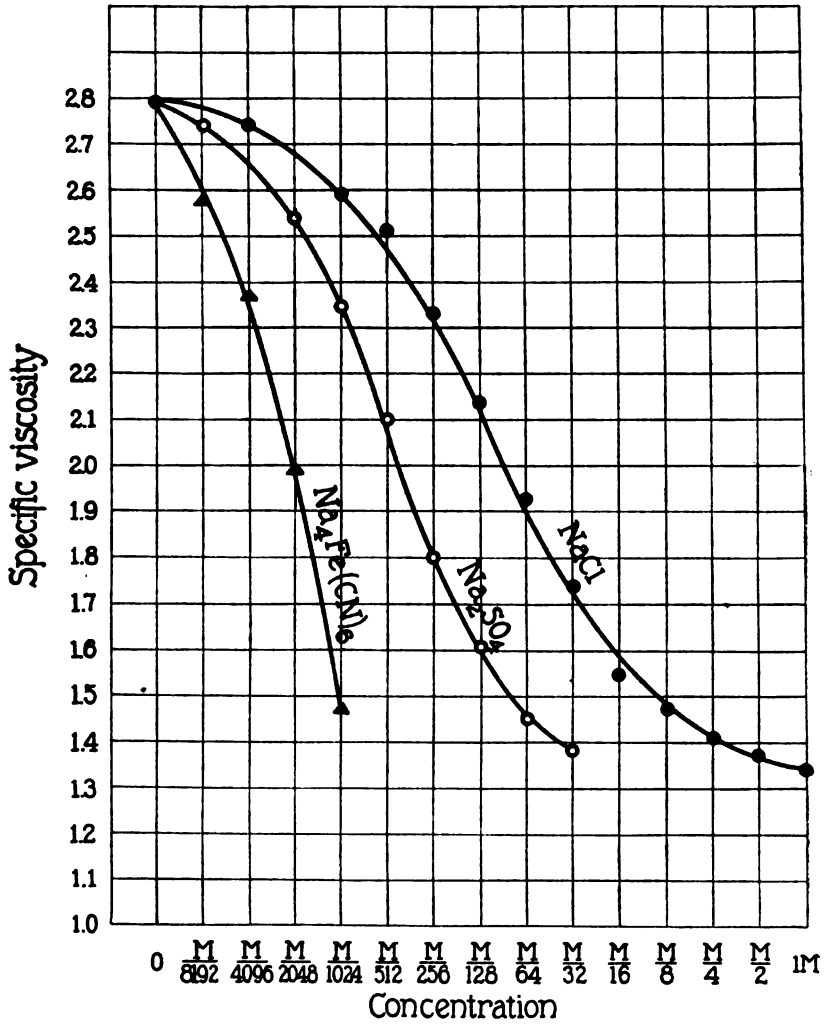

FIG. 6. The relative depressing effect of equal molecular concentrations of NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ on the specific viscosity of a gelatin chloride solution of pH 3.0 is approximately as 1:4:16.

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viscosity in the same way as the addition of KCl (Fig. 7); while the addition of KOH to Na gelatinate of pH 4.8 to 8.0 increases the viscosity and the addition of KCl to Na gelatinate always depresses the viscosity. The depressing effect of salts on metal gelatinate is due to the cation of the electrolyte added, that of bivalent cations being greater than that of monovalent cations, while the valency of the anion has no effect.

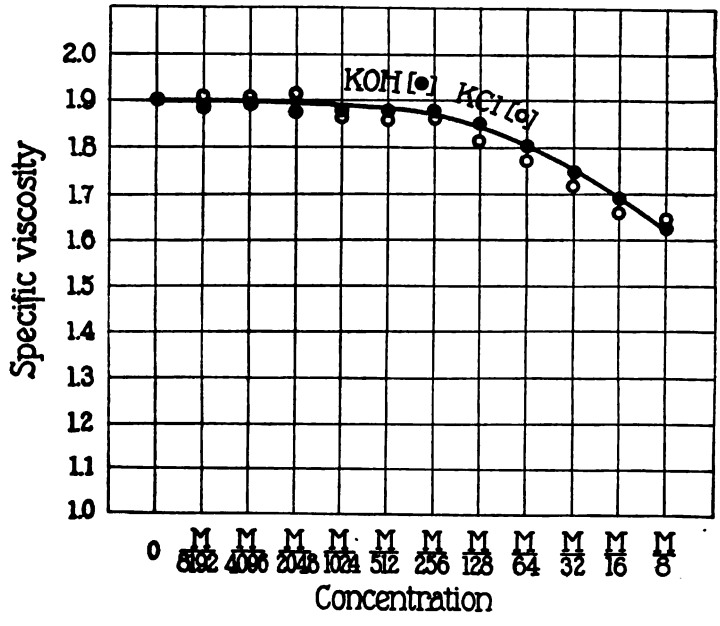

FIG. 7. The depressing effect of KOH and KCl on Na gelatinate of pH 12.0 is practically the same.

The addition of neutral salt to isoelectric gelatin leaves the viscosity and osmotic pressure of the solution practically unchanged.

The cause of this difference in the action of acids, alkalies, and salts is easy to understand on the basis of the purely chemical theory of the behavior of proteins. When we add acid to isoelectric gelatin we transform part of the latter into gelatin-acid salt and the proportion of gelatin-acid salt formed increases with the amount of the acid added. Near the isoelectric point the amount of gelatin-acid salt formed increases very rapidly with the addition of acid, but when

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the pH approaches 3.0 the addition of the same amount of acid which near the isoelectric point caused a considerable change now causes only a slight change, while when the pH falls below 3.0 the depressing influence of the anion continues to increase with increasing concentration of the electrolyte.

II. Ion Series and the Action of Salts on Proteins.

We have shown in preceding papers that, as long as the concentration of the electrolyte is not too high, only the sign of the charge and the valency of an ion influence such physical properties as swelling, viscosity, solubility in alcohol, and osmotic pressure of proteins; while all the different ions of the same sign of charge and valency have either the same effect or if there is a difference in effect it is too small to be noticed with our present methods of work.[5] This proof was furnished for the action of acids and alkalies on the physical properties of proteins just mentioned. We now wish to make the proof complete by considering also the action of salts. To do this we are compelled to compare the relative depressing action of low but equal concentrations of different salts upon the physical properties of a gelatin salt, for example gelatin chloride of a definite pH; *e.g.*, 3.0. As can be easily surmised the addition of a salt will in many cases alter the pH of the solution and this alteration will be larger in the case of certain salts, *e.g.* Na acetate, than in the case of others, *e.g.* NaCl. Unless we take into consideration these variations in the pH caused by the addition of salts we shall be in danger of erroneously ascribing the influence of a variation in the hydrogen ion concentration to an influence of the nature of the anion. The Hofmeister ion series are due to this error.

The method of our experiments was as follows. 50 cc. of a 2 per cent solution of originally isoelectric gelatin were brought to a pH of 3.0 by the addition of HCl. To this were added 50 cc. of H_2O or of a salt solution of different molecular concentration, and the viscosity of this mixture was measured using those precautions which were described in a preceding paper.

Fig. 8 gives the curves representing the depression of the specific viscosity of a gelatin chloride solution of pH 3.0 by different concentrations of salts with monovalent anion; namely, NaCl, NaH_2PO_4,

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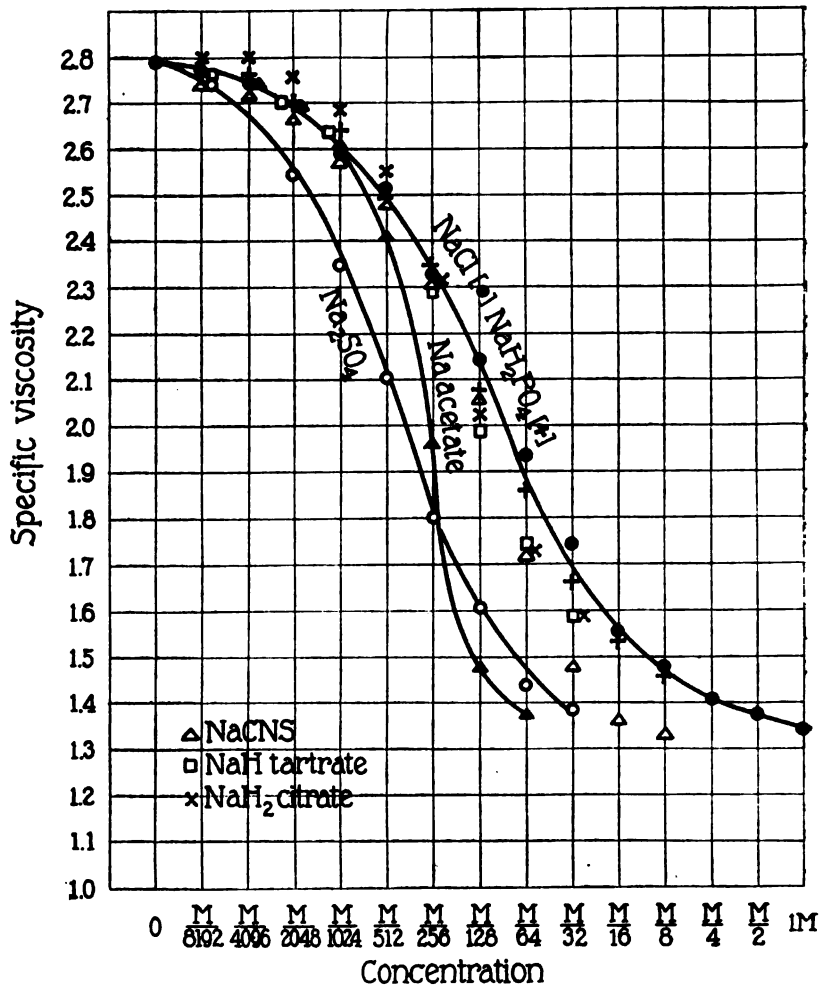

FIG. 8. The depressing effect of different salts with monovalent anion (NaCl, NaH_2PO_4, NaCNS, NaH tartrate, and NaH_2 citrate) on the specific viscosity of 1 per cent solution of gelatin chloride of pH 3.0. The effects of NaCl and NaH_2PO_4 are identical since the pH is not altered by the addition of these salts. The depression in the values for the specific viscosity is greater in the case of Na acetate than in the case of NaCl for the reason that the Na acetate raises the pH of the gelatin solution.

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NaCNS, NaH tartrate, NaH_2 citrate, and Na acetate. The curve for Na_2SO_4 is added for comparison. The monosodium salts of weak dibasic and tribasic acids dissociate electrolytically into a Na ion and a monovalent anion, H_2PO_4, H tartrate, H_2 citrate, etc. All the salts mentioned in Fig. 8 are therefore salts with monovalent anion with the exception of Na_2SO_4. Our valency rule demands that the relative depressing effect of these salts (with the exception of Na_2SO_4) should be the same and that deviations from this rule should find their explanation in corresponding deviations of the pH due to the influence of certain of the salts. We will first consider this latter influence as given in Table I, which shows the results of the measurements of pH

TABLE I.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.0 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.												
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
NaCl	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Na_2SO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.05	3.1	3.2	3.3	3.35
NaH_2PO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.3	3.4	3.45	3.5
NaCNS	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.3	3.6	3.9	4.2	4.4
NaH tartrate	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.3	3.45	3.5	3.55		
NaH_2 citrate	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.4	3.6	3.7	3.75		
Na acetate	3.0	3.0	3.0	3.05	3.1	3.3	3.7	4.3	4.6				

in these different gelatin solutions after the addition of salts. The original gelatin chloride solution had a pH of about 3.0 and this was not altered by the addition of NaCl and only slightly by the addition of NaH_2PO_4 in concentrations below M/16. According to the valency rule the curves for the depressing effect of NaCl and NaH_2PO_4 should be almost identical and Fig. 8 shows that this is the case.

Table I shows that NaCNS, monosodium tartrate, and monosodium citrate raise the pH of the solution as soon as the concentration reaches M/128 or more. If we consider this effect, we must expect to find that the drop in the curves for NaCNS, monosodium citrate, and monosodium tartrate is a little steeper in concentrations of M/128 and

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above than the curve for the depressing effect of NaCl. Fig. 8 shows that the curves for the depressing effect of these three salts are slightly lower than the curve for NaCl or NaH_2PO_4. The greatest apparent deviation from the valency rule occurs in the curve for Na acetate whose depressing effect is of the order of that of Na_2SO_4.

In the colloidal literature it is always stated that Na acetate acts like Na_2SO_4 and this is interpreted to mean that the acetate anion acts like the bivalent SO_4 anion and not like the monovalent Cl or NO_3 anion. Table I shows that Na acetate also depresses the hydrogen ion concentration more than NaCl or NaH_2PO_4; M/64 Na acetate brings the gelatin solution practically to the isoelectric point, and at the isoelectric point the viscosity of gelatin solution is a minimum. This lowering of the hydrogen ion concentration (and not the alleged influence of the acetate anion) explains the excessive depressing effect of Na acetate. That this interpretation is correct can be proved in the following way. We prepare 1 per cent solutions of gelatin acetate of pH 3.3 and gelatin chloride also of pH 3.3. The specific viscosity of these two solutions was practically the same (both were 1 per cent solutions in regard to originally isoelectric gelatin). The solution of gelatin acetate of pH 3.3 was made up in various concentrations of Na acetate of pH 3.3. The Na acetate solution of pH 3.3 was obtained by dissolving M/16 Na acetate in 1½ M acetic acid and the various degrees of dilution of this M/16 Na acetate solution of pH 3.3 were brought about by dilution with pure acetic acid of pH 3.3. The non-dissociated molecules of acetic acid have no more depressing influence on the physical properties of proteins than have the molecules of any non-electrolyte. Fig. 9 gives the curve representing the depressing effect of Na acetate on gelatin acetate of pH 3.3 when the pH is kept constant.

The gelatin chloride solution of pH 3.3 was made up in different concentrations of NaCl and the depressing effect of NaCl on the viscosity of gelatin chloride is also plotted in Fig. 9. It is obvious from Fig. 9 that the depressing effect of Na acetate and NaCl are identical when the pH is kept constant and identical in both cases.

The same fact was confirmed in a somewhat different way. A 2 per cent solution of gelatin chloride of pH 3.0 was made up in various concentrations of Na acetate also of pH 3.0. In order to prepare Na

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acetate solutions of pH 3.0 M/4 Na acetate was dissolved in M/4 HCl and the various dilutions required for the experiment were obtained by diluting the mixture of equal parts of M/4 HCl and M/4 Na acetate with M/1,000 HCl.

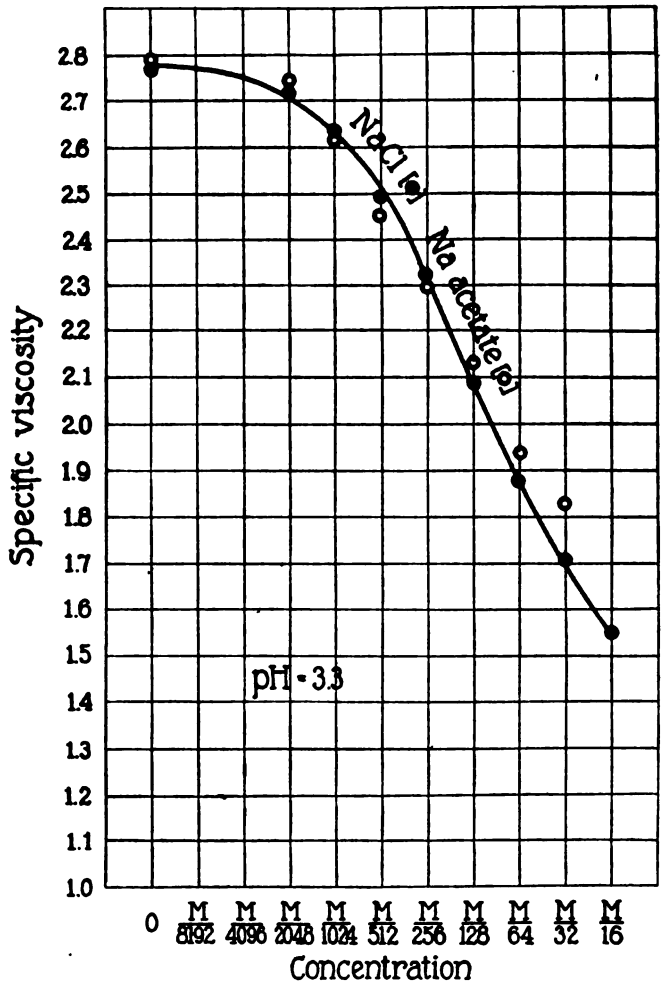

FIG. 9. When the pH is kept equal the depressing effect of equal concentrations of NaCl and Na acetate on the specific viscosity of a 1 per cent gelatin chloride or gelatin acetate solution of pH 3.3 is the same.

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The 2 per cent gelatin chloride solution of pH 3.0 was diluted with 50 cc. of this mixture so that the resulting 1 per cent gelatin chloride solution of pH 3.0 contained various concentrations of Na acetate (or

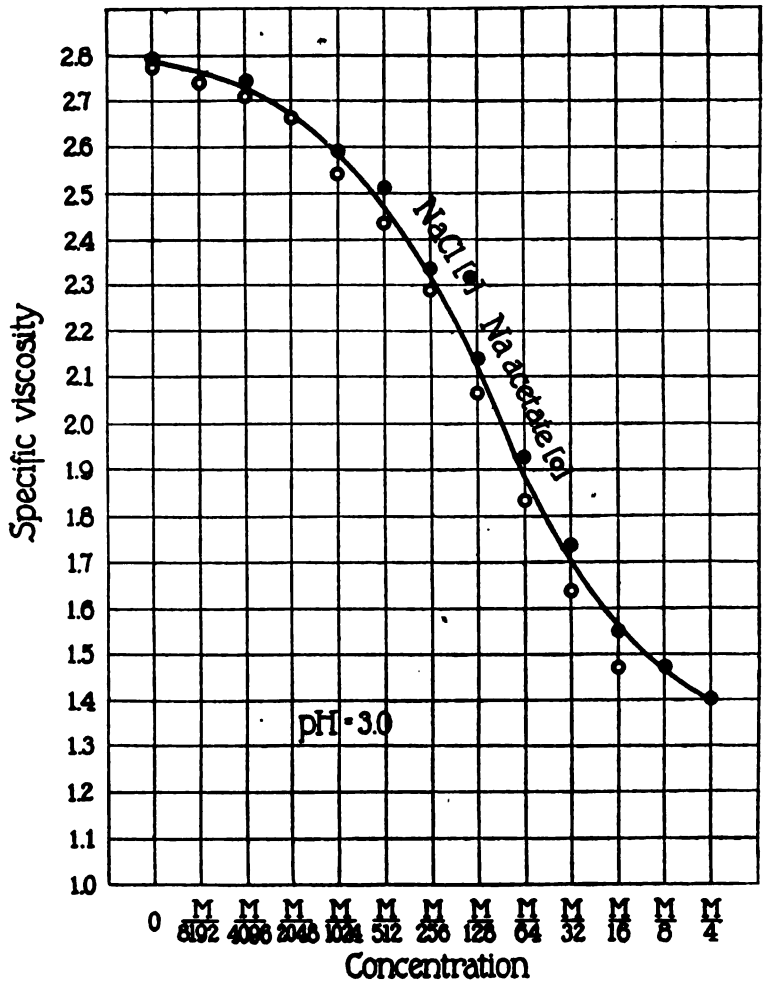

FIG. 10. See legend of Fig. 9, except that the pH of gelatin solution is 3.0.

more correctly of NaCl and Na acetate). The curve representing the depressing effect of this salt is given in Fig. 10, and is shown to be identical with the curve representing the depressing effect of the addition of NaCl to gelatin chloride of pH 3.0.

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We can, therefore, state that sodium acetate has the same effect on the viscosity of gelatin chloride as the addition of any other salt with monovalent anion, and that the anomalous effect ascribed to the acetate anion in the colloidal literature is in reality due to the depression of the hydrogen ion concentration of the gelatin solution by the Na

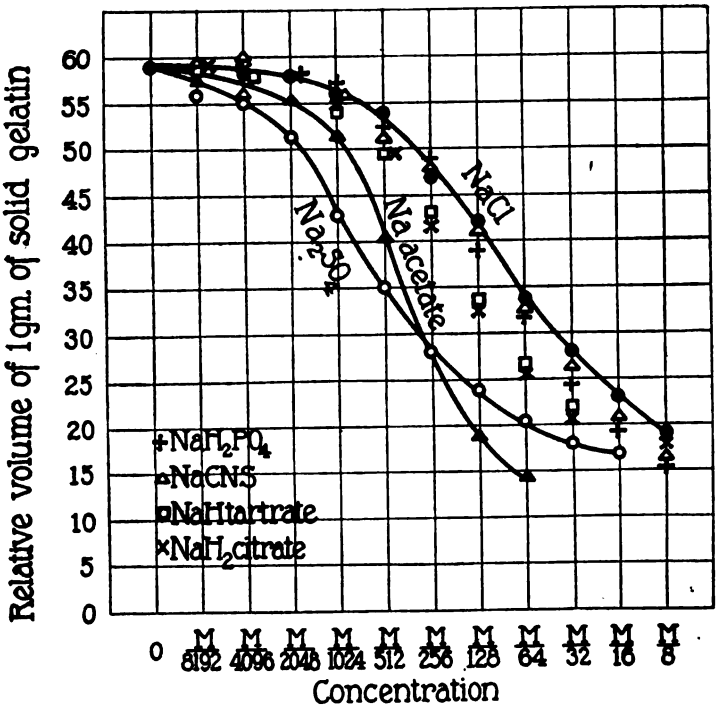

FIG. 11. Showing that the depressing effect of salts with monovalent anion on the swelling of gelatin chloride of pH 3.3 is similar to that on the specific viscosity. All salts with monovalent anion depress the swelling of gelatin chloride to the same extent, the seeming deviation from this rule being due to variation in the pH of the gelatin solution caused by buffer salts.

acetate which is a buffer salt. The failure to recognize the buffer character of salts, like the acetates, citrates, and tartrates, has led to the error of the Hofmeister ion series. In reality we find our valency rule confirmed whereby all salts with an anion of the same valency have the same relative depressing effect on the viscosity of a gelatin chloride solution if the pH of the solution is kept constant.

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What has been demonstrated for the effect of these salts on the viscosity of gelatin solutions holds also for their effect on the swelling of gelatin. The same volumetric method for measuring the swelling effect was used which was described in the preceding paper. Fig. 11 gives the relative depressing effect of NaCl, NaH_2PO_4, NaCNS, monosodium tartrate, monosodium citrate, and Na acetate on the swelling of gelatin chloride of pH 3.3 (the curve for Na_2SO_4 is added for comparison), and Table II gives the variation of the pH of the gelatin caused by the addition of these salts. Our theory demands that all these salts (except Na_2SO_4) should depress the swelling of gelatin chloride of pH 3.3 to the same amount, and that deviations from this

TABLE II.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.3 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
NaCl	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Na_2SO_4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.35	3.4	3.5	3.6
NaH_2PO_4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.6	3.7
NaCNS	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.35	3.4
NaH tartrate	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.5	3.6	3.7	3.7	3.7
NaH_2 citrate	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.6	3.8	3.85	3.9	3.9
Na acetate	3.3	3.3	3.3	3.4	3.45	3.5	3.8	4.3	4.8	5.2	5.4	5.5

rule must find their explanation in variations of pH caused by the addition of salt. Table II shows that the variations in pH are small for NaCl, NaCNS, and NaH_2PO_4 and hence the curves for the depressing effect of these three salts upon the swelling of gelatin are almost identical, as the valency rule demands. Monosodium citrate and tartrate have a greater depressing effect on the hydrogen ion concentration and Na acetate has a still greater depressing effect than these two salts. This explains the apparent deviation of the curves for these three salts from the valency rule.

Salts like disodium tartrate, disodium oxalate, and trisodium citrate offer an opportunity for an interesting test for our theory on account

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of the difference between the electrolytic dissociation of weak dibasic or tribasic acids and the salts of the same acids. A weak dibasic

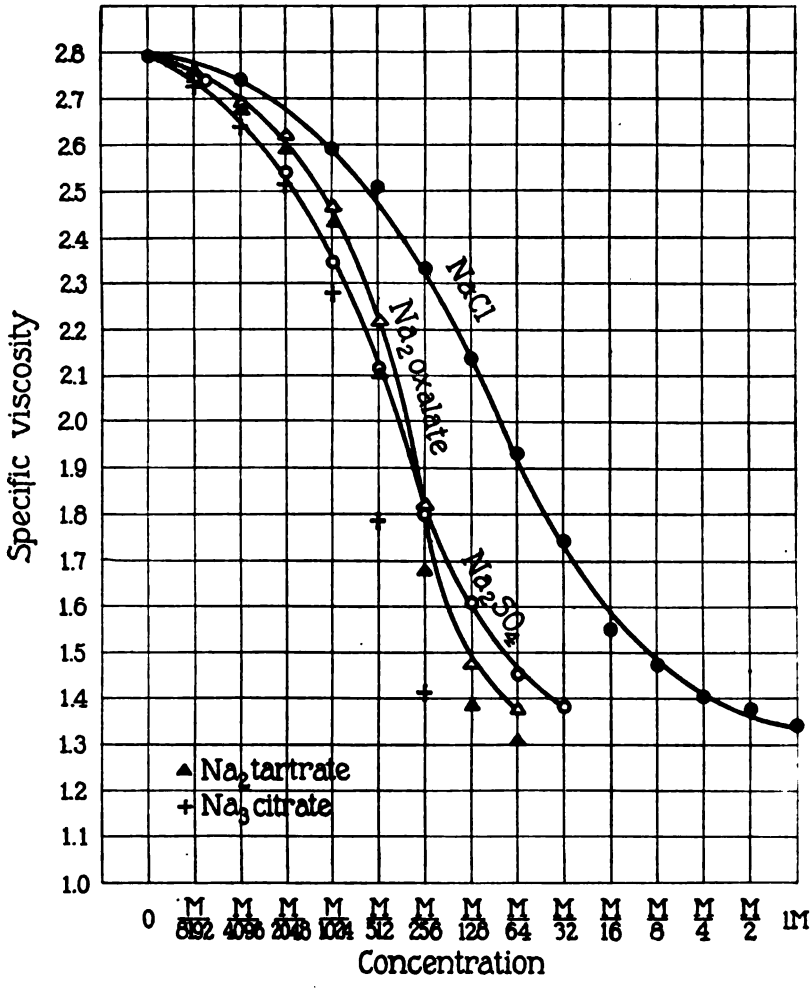

FIG. 12. Bivalent anions have an equally depressing effect as SO_4 on the specific viscosity of a 1 per cent gelatin chloride solution of pH 3.0.

acid like tartaric gives off one hydrogen ion easily but not both; sodium tartrate gives off both Na ions more readily. This is comprehensible on the idea that the oppositely charged ions in a molecule

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are held together by electrostatic forces and that these forces are stronger in the case of a hydrogen ion which is free from electrons than in the case of a Na ion where the positive nucleus is separated by two shells of electrons from the valency electron of the oppositely charged ions by which it is held. For this reason the tartaric acid, or the oxalic acid, anion combines with proteins in the form of a monovalent acid tartrate ion while we should expect the anion of disodium tartrate or disodium oxalate to be a bivalent anion. Hence the tartrate and oxalate anions should act like the sulfate anion when disodium tartrate or disodium oxalate are added to a protein solution. This is confirmed, as Fig. 12 shows. The curves for the depressing effect of Na_2 oxalate and Na_2 tartrate practically coincide with the curve for

TABLE III.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.0 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.												
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
NaCl	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Na_2SO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.05	3.1	3.2	3.3	3.35
Na_2 oxalate	3.0	3.0	3.0	3.05	3.1	3.3	3.5	3.9	4.2	4.4			
Na_2 tartrate	3.0	3.0	3.0	3.1	3.2	3.3	3.7	4.0	4.35	4.7	4.8	5.0	
Na_3 citrate	3.0	3.0	3.05	3.2	3.3	3.8	4.4	5.2	5.7	6.1			

the depressing effect of Na_2SO_4 on the viscosity of gelatin solution, except in concentrations greater than M/256 where disodium tartrate and disodium oxalate cause also a depression of the hydrogen ion concentration (Table III) and where therefore the curves for these two salts drop more rapidly than the curve for Na_2SO_4. Hence disodium oxalate and disodium tartrate act like Na_2SO_4, while, as Fig. 8 shows, monosodium tartrate, monosodium citrate, and monosodium phosphate act like NaCl (if the necessary corrections for pH are made).

The osmotic pressure, viscosity, and swelling of Na gelatinate should be depressed by the cation of a salt and the more so the higher the valency of the cation. Fig. 13 shows that this is true for the swelling of Na gelatinate of pH about 9.3. The molecular concentration in

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which the swelling is depressed by the same amount is about half as great for Na_2SO_4 as for NaCl (for molecular concentrations from M/256 to M/32), proving that the Na ion is responsible for the depression, while it is about eight times as high for NaCl as for $CaCl_2$. The pH of the gelatin was practically the same in all solutions.

All these data confirm our valency rule, whereby ions of the same valency and the same sign of charge have, in the same concentration, the same depressing effect on osmotic pressure, swelling, solubility in

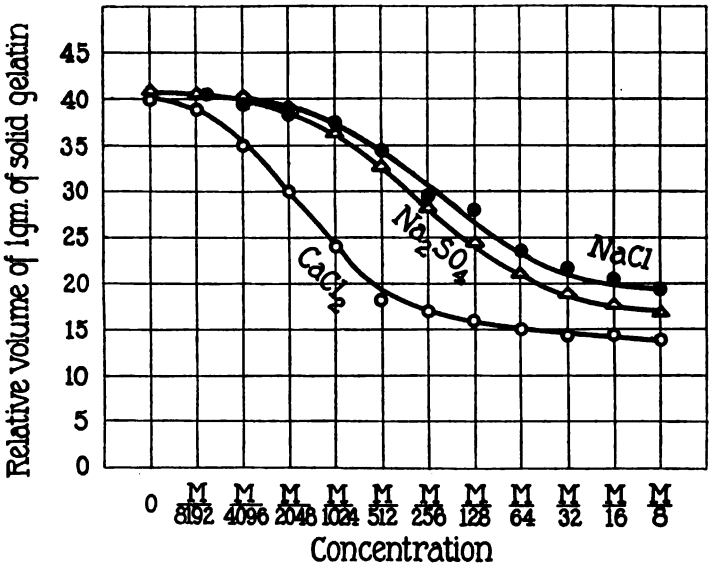

FIG. 13. The depressing effect of neutral salts on the swelling of Na gelatinate of pH about 9.3 is due to the cation of the salt, the depressing effect of NaCl being half as great as that of Na_2SO_4 of equal molecular concentration of Na_2SO_4 while that of $CaCl_2$ is considerably greater owing to the fact that Ca is bivalent.

alcohol, and viscosity of proteins; while the depressing effect increases rapidly with the valency. The Hofmeister ion series are due to the neglect of measuring the influence of the salts on the hydrogen ion concentration of the gelatin solutions. This neglect has given rise to the statement that salts, like sodium acetate, have the same depressing effect on the physical properties of proteins as the sulfates. A number of attempts to apply colloid chemistry to biology, pathology, and medicine are based on such errors.

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SUMMARY.

1. Ions with the opposite sign of charge as that of a protein ion diminish the swelling, osmotic pressure, and viscosity of the protein. Ions with the same sign of charge as the protein ion (with the exception of H and OH ions) seem to have no effect on these properties as long as the concentrations of electrolytes used are not too high.

2. The relative depressing effect of different ions on the physical properties of proteins is a function only of the valency and sign of charge of the ion, ions of the same sign of charge and the same valency having practically the same depressing effect on gelatin solutions of the same pH while the depressing effect increases rapidly with an increase in the valency of the ion.

3. The Hofmeister series of ions are the result of an error due to the failure to notice the influence of the addition of a salt upon the hydrogen ion concentration of the protein solution. As a consequence of this failure, effects caused by a variation in the hydrogen ion concentration of the solution were erroneously attributed to differences in the nature of the ions of the salts used.

4. It is not safe to draw conclusions concerning specific effects of ions on the swelling, osmotic pressure, or viscosity of gelatin when the concentration of electrolytes in the solution exceeds M/16, since at that concentration the values of these properties are near the minimum characteristic of the isoelectric point.

Note.—The solutions of 1 per cent isoelectric gelatin were prepared by bringing 1 gm. of dry gelatin to the isoelectric point according to the method described in previous papers. It was found that in this process about 20 per cent of the gelatin was lost, so that the originally 1 per cent gelatin solution contained in reality only about 0.8 per cent isoelectric gelatin. This does not affect the contents of the paper or the conclusions since the gelatin concentration used was always the same.

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A THEORY OF INJURY AND RECOVERY.

II. Experiments with Mixtures.

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(Received for publication, January 15, 1921.)

The electrical conductivity of *Laminaria* changes when the plant is transferred from sea water to 0.52 M NaCl, or to 0.278 M $CaCl_2$, and is subsequently replaced in sea water. It has been found that these changes can be predicted with considerable accuracy by means of certain equations. According to the theory of the writer,[1] the same equations should enable us to predict the changes produced by transferring tissue from sea water to mixtures of NaCl and $CaCl_2$ and then replacing the plant in sea water.

In order to test this theory experiments were made with a variety of mixtures. The solutions employed are given in Table I. The electrical resistance of the tissue in these solutions is shown in Fig. 1.

The experimental data are in good agreement with the values calculated by means of the formula:

$$\text{Resistance} = 2{,}700\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 90\, e^{-K_M T} + 10$$

This is evident from Fig. 1, which shows the curves calculated by means of this formula and also the observed values.[2]

[1] Osterhout, W. J. V. *J. Gen. Physiol.*, 1920-21, iii, 145. For corrections see the slips inserted in this and in the preceding number of the *Journal*.

[2] The points shown in Fig. 1 (except those for the solutions containing 1.41 and 2.44 per cent $CaCl_2$) are taken from Fig. 1 of a previous paper (cited below), but the curves here given are not identical with those shown in that figure since the curves of the present paper represent the calculated values (using the velocity constants given in Table II), while those in Fig. 1 of the former paper are merely smoothed curves drawn through the observed points.

The results described in the former paper as obtained with solutions containing

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TABLE I.

Composition of Mixtures.

0.52 M NaCl	0.278 M $CaCl_2$	Molecular proportions in the mixture.	
		NaCl	$CaCl_2$
cc.	*cc.*	*per cent*	*per cent*
973	27	98.59	1.41
955	45	97.56	2.44
914	86	95.24	4.76
751	249	85.00	15.00
496	504	65.00	35.00
247	753	38.00	62.00

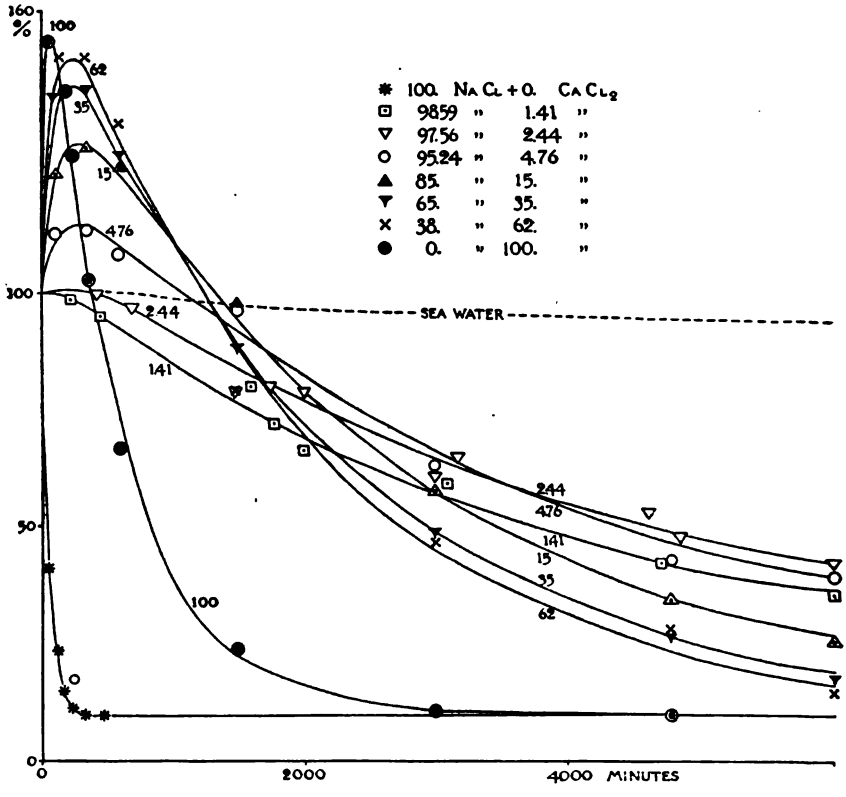

FIG. 1. Curves showing the electrical resistance of *Laminaria agardhii* in 0.52 M NaCl, in 0.278 M $CaCl_2$, and in mixtures of these (the figures attached to the curves show the molecular per cent of $CaCl_2$ in the solution). The curves show the calculated values (from constants obtained by trial, which are given in Table II); the points show the observed values (some are omitted in order to avoid undue crowding); each represents the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

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This formula is based upon the assumption that the electrical resistance is proportional to a substance, M, which is formed and decomposed by the reactions[3]

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$$

We assume that when the tissue is transferred from sea water to NaCl, or to $CaCl_2$, or to a mixture of these two solutions, the reactions $O \rightarrow S \rightarrow A$ cease, while the reactions $A \rightarrow M \rightarrow B$ continue. By assuming various values of K_A (the velocity constant of the reaction $A \rightarrow M$) and of K_M (the velocity constant of the reaction $M \rightarrow B$), and employing these in the formula, we obtain curves which closely approximate those which we find by experiment. The values of the velocity constants which are thus obtained are given in Table II.

It is evident from Table II that as the per cent of $CaCl_2$ in the mixtures increases (beginning at 1.41 per cent $CaCl_2$) the value of K_M first falls and then rises, its minimum value occurring in 97.56 NaCl + 2.44 $CaCl_2$ (which is the mixture in which the tissue lives the longest). It seems reasonable to assume that in each mixture a substance is formed which reduces the value of K_M. We may assume that the decrease of K_M is directly proportional to the amount of this substance, which may be assumed to occur in maximum amount in 97.56 NaCl + 2.44 $CaCl_2$.

The simplest assumption which we can make is that NaCl and $CaCl_2$ combine with some constituent of the protoplasm, as XZ_2, to form a compound.[4] If we suppose that the compound is Na_4XCa, formed by the reversible reaction

$$4\,NaCl + XZ_2 + CaCl_2 \rightleftharpoons Na_4XCa + 2\,ZCl_2$$

we can calculate the amount of Na_4XCa which is formed in each mixture of NaCl and $CaCl_2$.

2 per cent $CaCl_2$ are omitted, as it was subsequently found that this solution contained more than 2 per cent $CaCl_2$.

For the data concerned and for the derivation of the formula see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533. In the present paper the formula previously used is multiplied by 305 and 10 is added.

[3] These are regarded as monomolecular and irreversible or practically so.

[4] It is assumed that XZ_2, Na_4XCa, and ZCl_2 are in solution. Since the per cent of XZ_2 which is transformed to Na_4XCa is negligible, the concentration of XZ_2 may be regarded as constant.

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TABLE II.

Velocity Constants at 15°C.

Reaction.	Velocity constant.	Value of the velocity constant in*							
		NaCl	NaCl 98.59 $CaCl_2$ 1.41	NaCl 97.56 $CaCl_2$ 2.44	NaCl 95.24 $CaCl_2$ 4.76	NaCl 85.0 $CaCl_2$ 15.0	NaCl 65.0 $CaCl_2$ 35.0	NaCl 38.0 $CaCl_2$ 62.0	$CaCl_2$
$N \rightarrow O$	K_N	0.03	0.00046	0.0004	0.00061	0.00091	0.0013	0.00134	0.005033
$O \rightarrow P$	K_O	0.0297	0.0004554	0.000396	0.0006039	0.0009009	0.001287	0.0013266	0.0049827
$R \rightarrow S$	K_R	0.04998	0.0005623	0.00056	0.001055	0.001606	0.0026	0.003509	0.012532
$S \rightarrow T$	K_S	0.02856	0.0008048	0.000874	0.00151	0.0017446	0.00254	0.00319	0.010848
$A \rightarrow M$	K_A	0.018	0.000222	0.000187	0.000245	0.000364	0.000481	0.00053	0.0018
$M \rightarrow B$	K_M	0.540	0.00666	0.00546	0.00590	0.00730	0.00859	0.009	0.0295

* The figures at the heads of columns refer to molecular proportions in the mixture.

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For reasons given in a former paper,[5] we assume that this reaction occurs at the surface of the cell and that $CaCl_2$ accumulates in the surface to a greater degree than NaCl. The increase in concentration of $CaCl_2$ in the surface is supposed to be ten times as great as the corresponding increase of NaCl, so that the proportions in the surface are those given in Table III. For example, when the proportions in the solution are 97.56 NaCl + 2.44 $CaCl_2$, the proportion of NaCl to $CaCl_2$ in the surface is as 97.56 to 24.40, which is equivalent[6] to 80 NaCl + 20 $CaCl_2$.

TABLE III.

Amount of Na_4XCa.

Molecular proportions.				Amount of Na_4XCa	Increase in Na_4XCa
In the solution.		In the surface.			
NaCl	$CaCl_2$	NaCl	$CaCl_2$		
per cent	*per cent*	*per cent*	*per cent*		
100.0	0	100.0	0	0	0
98.59	1.41	87.5	12.5	0.000902	0.000855
97.56	2.44	80.0	20.0	0.000936	0.000889
95.24	4.76	66.67	33.33	0.000870	0.000823
85.0	15.0	36.27	63.73	0.000488	0.000441
65.0	35.0	15.66	84.34	0.000177	0.000125
38.0	62.0	5.78	94.22	0.000047	0
0	100.0	0	100.0	0	0

We calculate the amount of Na_4XCa by the usual formula:

$$K = \frac{(C_{Na_4XCa})\,(C_{ZCl_2})^2}{(C_{NaCl})^4\,(C_{CaCl_2})\,(C_{XZ_2})}$$

but since $2C_{Na_4XCa} = C_{ZCl_2}$ we may write

$$K = \frac{(C_{Na_4XCa})\,(2\,C_{Na_4XCa})^2}{(C_{NaCl})^4\,(C_{CaCl_2})\,(C_{XZ_2})}$$

[5] Osterhout, W. J., V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

[6] As explained in a former paper,[5] it is assumed that the reaction takes place in a surface which is saturated with respect to NaCl and $CaCl_2$, so that while one of these may be displaced by the other (in case their relative proportions in the solution are altered) the total concentration does not change; for convenience this concentration is taken as 100 and the sum of NaCl + $CaCl_2$ is therefore always equal to 100.

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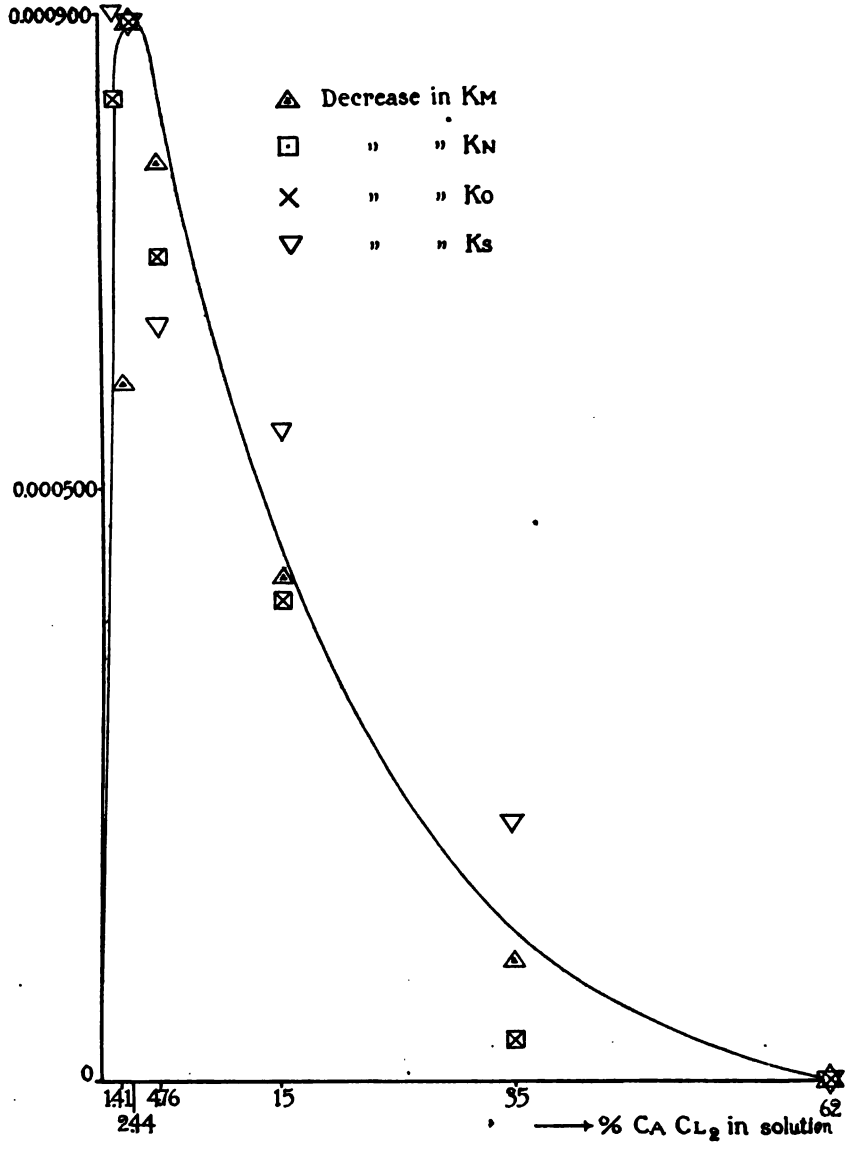

FIG. 2. Curve showing the increase of a hypothetical salt compound Na_4XCa (see Table III); and the corresponding decrease of the velocity constants K_N, K_O, K_S, and K_M (these constants are given in Table II). The figures on the abscissa give the molecular per cent of $CaCl_2$ in the mixture. The mixture containing 62.0 per cent $CaCl_2$ is taken as the standard of comparison: proceeding from this to the mixtures containing less $CaCl_2$ we find that Na_4XCa increases and the velocity constants decrease as shown by the ordinates. In order to facilitate comparison the values of K_N have been multiplied by 0.989; of K_O by 0.991; of K_S by 0.384; and of K_M by 0.251.

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Putting $K = 4(10^{-17})$ and $C_{XZ_4} = 0.1$ we get[4]

$$10^{-17} = \frac{(C_{Na_4XCa})^3}{(80)^4\ (20)\ (0.1)}$$

whence $C_{Na_4XCa} = 0.000936$.

Proceeding in the same manner with the other mixtures we get the values given in Table III. Starting with the lowest value (that in 62.0 per cent $CaCl_2$) we observe that there is an increase as the per cent of $CaCl_2$ decreases until 2.44 per cent is reached (the amount of this increase is shown in Column 6 of the table). Conversely we find (Table II) that the velocity constants are higher in 62.0 per cent $CaCl_2$ than in any other mixture and that they decrease as the per cent of $CaCl_2$ decreases to 2.44 per cent. Thus in the case of K_M the value in 62.0 per cent $CaCl_2$ is 0.009, in 2.44 per cent $CaCl_2$ it is less by 0.00354, while in 15.0 per cent it is less by 0.0017, and in 35.0 per cent by 0.00041; if we multiply these numbers by the constant factor 0.251 they agree very closely with the figures for the increase in Na_4XCa. These values are plotted in Fig. 2, which shows that the decrease in K_M is directly proportional to the increase in the amount of Na_4XCa. Hence we assume that Na_4XCa acts as a negative catalyzer or inhibitor of the reaction $M \rightarrow B$.

An inspection of Table II shows that the value of K_A fluctuates with that of K_M except that as $CaCl_2$ increases the value of K_A rises more rapidly than that of K_M. This is also obvious from Fig. 1, which shows that the greater the per cent of $CaCl_2$ in the mixture the greater the maximum attained. Since this maximum increases as the value of $K_A \div K_M$ increases, it is evident that the value of $K_A \div K_M$ must rise as the per cent of $CaCl_2$ becomes greater. The value of $K_A \div K_M$ in the solution containing 1.41 per cent of $CaCl_2$ is 0.03333 while in the solution containing 62.0 per cent $CaCl_2$ it is 0.05889, an increase of 0.02556. If we calculate this increase for the other mixtures and plot the values so obtained against the per cent of $CaCl_2$ in the surface, we obtain a straight line as shown in Fig. 3. This indicates that $CaCl_2$ catalyzes the reaction $A \rightarrow M$; for if this were not the case the value of K_A and K_M would rise and fall in such a way that the value of $K_A \div K_M$ would remain constant.

It is evident from Figs. 2 and 3 that the values of K_A and K_M are determined by the amount of Na_4XCa and by the per cent of $CaCl_2$

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in the mixture, and that when these values are experimentally determined for any two mixtures they can be calculated for any other mixture. When this is done we can calculate the course of the death curve in that mixture.

Having thus accounted for the death curves, we may turn our attention to the process of recovery. We find that, when tissue is removed from a mixture of NaCl and $CaCl_2$ and replaced in sea water, the resistance at once rises or falls and after a time becomes stationary. This rise or fall of resistance may be called recovery.

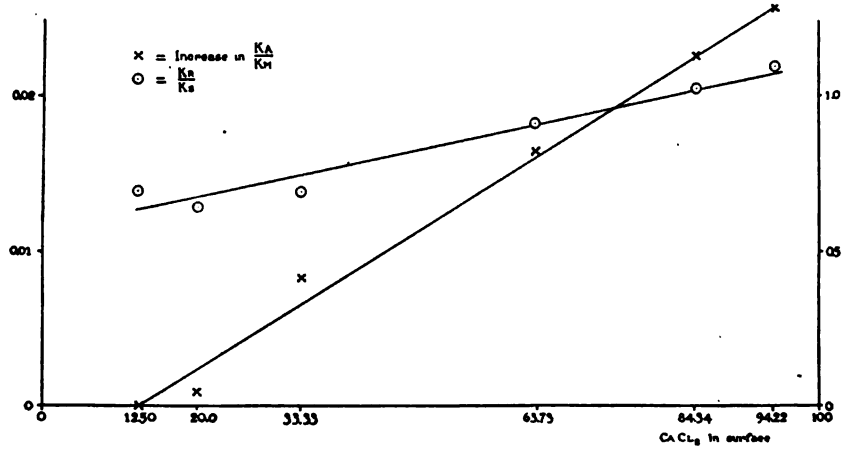

FIG. 3. Graph showing the increase of $K_A \div K_M$ and the value of $K_R \div K_S$ as the molecular per cent of $CaCl_2$ increases. The figure shows that $CaCl_2$ acts as a catalyzer of the reaction $A \rightarrow M$ (which has the velocity constant K_A) and also of the reaction $R \rightarrow S$ (which has the velocity constant K_R). The figures on the ordinate at the right show the values of $K_R \div K_S$; those on the ordinate at the left show the increase in the value of $K_A \div K_M$ over the value found in the mixture containing 1.41 per cent $CaCl_2$. The abscissæ denote molecular per cent of $CaCl_2$ in the surface (not in the solution).

In order to account for the facts we suppose that when we replace the tissue in sea water the reactions $O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$ proceed at the rates which are normal for sea water. The manner in which the rate of recovery is calculated has been explained in detail in a previous paper.[1] It is assumed that during the exposure to any of the mixtures the following reactions occur: (1) $N \rightarrow O \rightarrow P$; (2) $R \rightarrow S \rightarrow T$; (3) $A \rightarrow M \rightarrow B$. By assuming values of the velocity

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ts of these reactions we can approximate the observed results. ocity constants thus found are given in Table II. An inspec- the table shows that all these velocity constants behave like K_M in that as the per cent of $CaCl_2$ in the mixture increases ing with 1.41 per cent $CaCl_2$) the value of the velocity con- rst falls and then rises, and that this value in every case reaches imum in the mixture containing 97.56 NaCl + 2.44 $CaCl_2$. ld therefore appear that the reactions $N \rightarrow O \rightarrow P$ and $R \rightarrow$ are inhibited by Na_4XCa in the same manner as the reactions $A \rightarrow B$. This is borne out by an inspection of Fig. 2. in which crease[7] of the velocity constants is plotted, together with the e of Na_4XCa.

have seen that the value of $K_A \div K_M$ increases as the per cent Cl_2 increases and we interpreted this to mean that the reaction A is catalyzed by $CaCl_2$. In the same manner we infer that action $R \rightarrow S$ is catalyzed by $CaCl_2$, since we find that the of $K_R \div K_S$ increases with increasing percentage of $CaCl_2$, as in Fig. 3. It is not certain that the curve does not reach a um in the mixture of 97.56 NaCl + 2.44 $CaCl_2$ but for prac- urposes we may, for the present, regard it as a straight line.[8]

the decrease in the velocity constant is meant the decrease which we as we pass from the solution containing the highest per cent of calcium er cent NaCl + 62.0 per cent $CaCl_2$) to mixtures containing smaller per f calcium. Thus the decrease of $K_M = 0.009 - K_M$; the decrease of $0.00134 - K_N$; the decrease of $K_O = 0.0013266 - K_O$; and the decrease $= 0.00319 - K_S$. In the same manner we find that the increase in the t of Na_4XCa = amount of $Na_4XCa - 0.000047$.

decrease of the amount of K_A and K_R is not shown in the figure because nds not only on Na_4XCa but also on the per cent of $CaCl_2$.

fact that even in the presence of the maximum amount of Na_4XCa these y constants are greater than in sea water is of course to be attributed to the ubstances present in sea water.

ace in pure NaCl or $CaCl_2$ the salt compound Na_4XCa is not formed, we expect that in these solutions all the reactions would be more rapid than mixtures. That this expectation is fully realized is evident from Table II. velocity constants are somewhat higher in NaCl than in $CaCl_2$; this is plained by the assumptions already made but it does not seem desirable at t to make additional assumptions for this purpose. We might expect the of $K_A + K_M$ and $K_R \div K_S$ to reach a maximum in $CaCl_2$. This is actually se. It might perhaps be expected that these values would fall to a minimum Cl. This is the case with $K_A \div K_M$ but not for $K_R \div K_S$.

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The relation between K_N and K_O is taken as constant in the proportion of 100 to 99.

It is evident that when the constants have been empirically determined for two mixtures the constants for any other mixture can be calculated at once, since all of them depend in a definite manner on Na_4XCa (K_A and K_R also depend on the per cent of $CaCl_2$). The agreement between the constants thus obtained by calculation and those found by trial is fairly close, as is evident from Figs. 2 and 3.[9]

It has been shown in a previous paper that the height to which the recovery curve rises depends on the value of *O:* the value of *O*

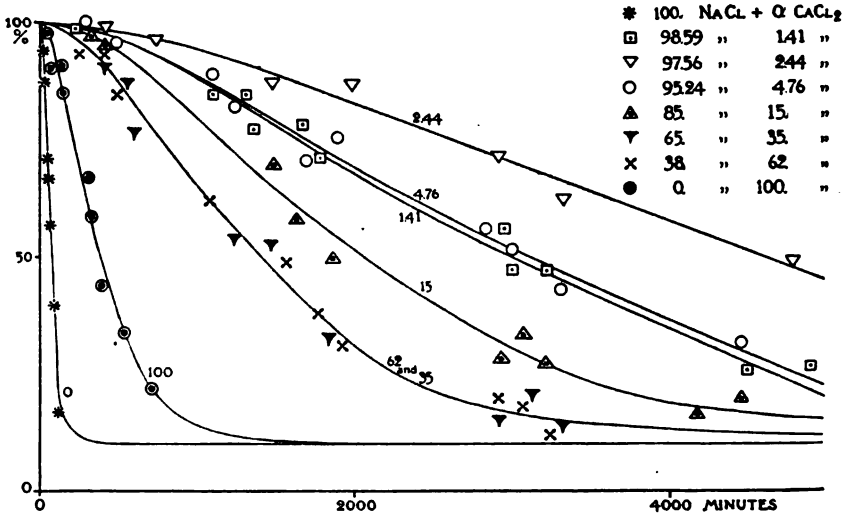

FIG. 4. Curves showing the value of $O + 10$ in 0.52 M NaCl, in 0.278 M $CaCl_2$, and in mixtures of these (the figures attached to the curves show the molecular per cent of $CaCl_2$ in the solution). The ordinates give the relative values of $O + 10$, the value in sea water being arbitrarily taken as 100 per cent. These values are obtained by exposing tissue to toxic solutions and then finding the level to which the resistance rises or falls after the tissue is replaced in sea water: they are therefore a measure of permanent injury. The abscissæ give the length of exposure to the toxic solution. The curves show the calculated values (using the velocity constants given in Table II). The points show the observed values; each represents the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

[9] The constants obtained by calculation would fall exactly on the graphs in these figures while those found by trial are indicated by the points given.

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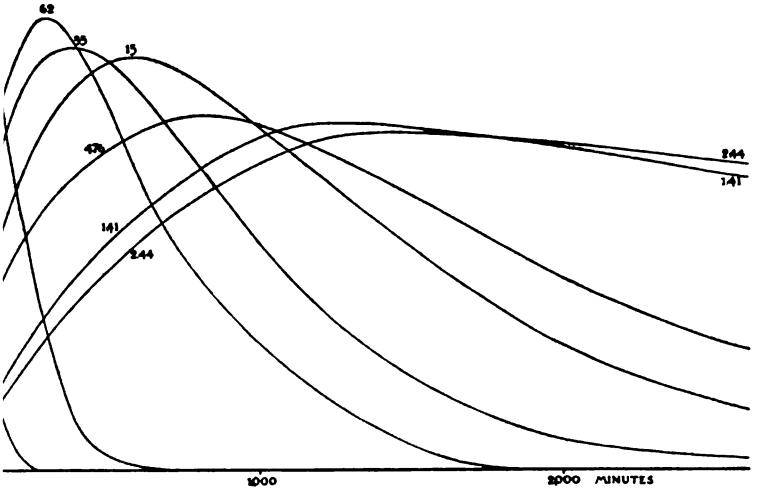
; shown for all the solutions[10] in Fig. 4, which shows the agree- between observation and calculation in respect to the final ached by the recovery curve, but not in respect to speed of y, which depends more on the value of S than on that of O. te of recovery seems to be about the same in the mixtures as pure salts. In general it is found that the rise or fall is nine- completed in about an hour.

5 shows the calculated values of S; observed values are not because they cannot be very precisely determined. This is



. 5. Curves showing the (calculated) values of S in 0.52 M NaCl, 0.278 M , and in mixtures of these (the figures attached to the curves show the ılar strength of $CaCl_2$ in the solution). The curves show the values cal- d from constants obtained by trial, which are given in Table II. The ıæ represent the time of exposure to the toxic solution. The value of S start is in all cases 2.7.

---

he values of $O + 10$ for solutions containing 2.44 and 15.0 per cent $CaCl_2$ slightly from those given in a former paper (Osterhout, W. J. V., *J. Gen.* ol., 1920–21, iii, 15) for the reason that the curves here presented include a series of experiments. 10 is added to the value of O because the base line en as 10, just as in the case of M.

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owing to the fact that S affects only the speed of recovery (not the final level attained) and as the speed is variable the most satisfactory procedure is to assume such values of K_R and K_S in the equation[11]

$$S = R\left(\frac{K_R}{K_S - K_R}\right)\left(e^{-K_R T} - e^{-K_S T}\right) + S_0 e^{-K_S T}$$

as cause the closest approximation to the observed speed of recovery. The values of S thus obtained for each solution are shown in the figure. In general the speed of recovery, as calculated from these values of S, is in satisfactory agreement with the observations.

By means of the equations given in the previous paper, and of the velocity constants in Table II of this paper, we are able to calculate the recovery curves for any solution after any length of exposure.

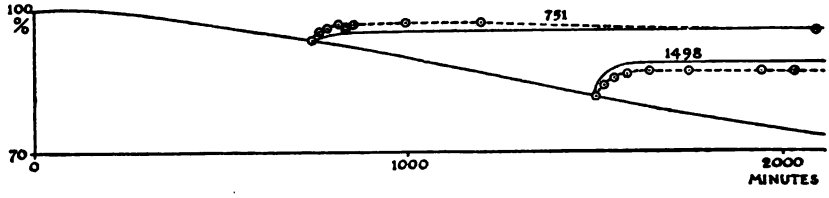

FIG. 6. Curves showing the electrical resistance (descending curve) of *Laminaria agardhii* in a mixture containing 97.56 mols of NaCl to 2.44 mols of $CaCl_2$ and recovery in sea water (ascending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the toxic solution. In the recovery curves the experimental results are shown by the broken lines, the calculated results by the unbroken lines. The observed points represent the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

Lack of space prevents a tabulation of the observed and calculated values, but it is possible to exhibit graphically the data for three mixtures and for this purpose one in which recovery consists in a rise of resistance (Fig. 6), one in which it shows a moderate fall (Fig. 7), and one showing a very decided fall (Fig. 8) are presented. In general the agreement between observation and calculation is satisfactory for all the solutions employed in the investigation.

It might be thought that the number of constants is sufficient to make it possible to fit any sort of experimental curve and that the

[11] *Cf.* equation (3) of the preceding paper (Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21. iii, 145).

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consequent agreement between observed and calculated results is less significant than would otherwise be the case. But, as a matter of fact, the constants are so related to each other and to the salt compound, Na_4XCa that the whole set of curves fits into a consistent scheme, so that when the constants are determined for any two mixtures the theoretical curves for all the other mixtures are thereby fixed. Under these circumstances the close agreement in the six different mixtures (ranging from 1.41 to 62.0 per cent $CaCl_2$) seems to be significant.

There seems to be no doubt that the behavior of the tissue is such as to indicate an underlying mechanism which is the same in all cases.[12] We have assumed that this mechanism consists in the production and decomposition of a substance, M, the amount of which, in the mix-

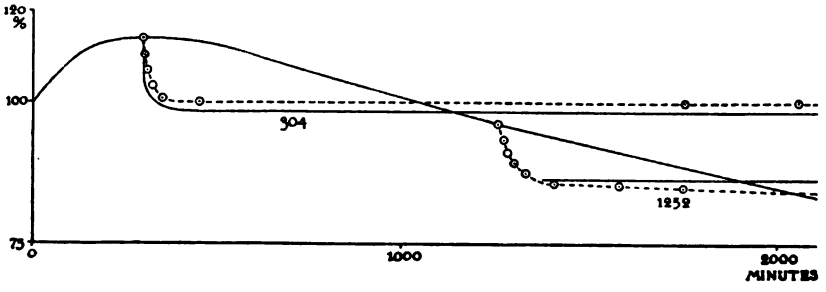

FIG. 7. Curves showing the electrical resistance (curve which ascends and descends) of *Laminaria agardhii* in a mixture containing 95.24 mols of NaCl to 4.76 mols of $CaCl_2$, and recovery in sea water (descending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the toxic solution.

In the recovery curves the experimental results are shown by broken lines, the calculated results by unbroken lines. The observed points represent the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

[12] This is shown, for example, by the fact that the rapidity of permanent injury (as observed after replacement in sea water) corresponds throughout with the rate of death, and that the rate of change of M corresponds throughout with the rate of change of O, S, and A. In other words if we change the solution in such a way as to increase (or decrease) the rate of one of the reactions on which the resistance depends we simultaneously increase (or decrease) the rates of all the others in a definite and predictable manner.

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tures, depends largely on a compound Na_4XCa formed by the combination of Na and Ca with a constituent X of the protoplasm. It is not necessary to discuss these asumptions more fully at present. But it may be pointed out that two things seem to be fairly well established; (1) a consistent mechanism underlies the entire behavior of the

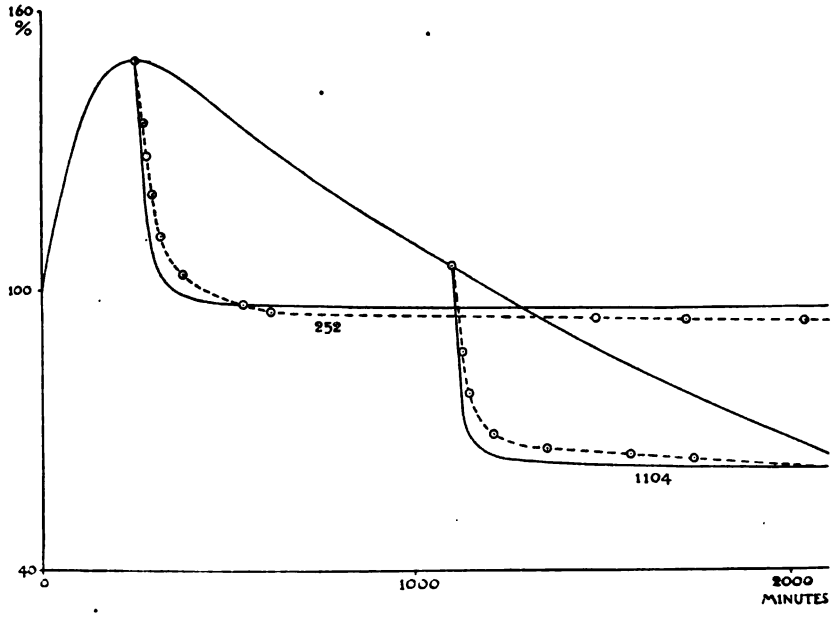

FIG. 8. Curves showing the electrical resistance (curve which ascends and descends) of *Laminaria agardhii* in a mixture containing 38 mols of NaCl to 62 mols of $CaCl_2$, and recovery in sea water (descending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the toxic solution. In the recovery curves, the experimental results are shown by the broken lines, the calculated results by the unbroken lines. The observed points represent the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

tissue, and (2) its operation can be predicted with a fair degree of accuracy by means of the equations which have been developed. The predictive value of these equations may be regarded as permanently established, since it does not depend on our views regarding the underlying assumptions.

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SUMMARY.

1. The equations which serve to predict the injury of tissue in 0.52 M NaCl and in 0.278 M $CaCl_2$ and its subsequent recovery (when it is replaced in sea water) also enable us to predict the behavior of tissue in mixtures of these solutions, as well as its recovery in sea water after exposure to mixtures.

2. The reactions which are assumed in order to account for the behavior of the tissue proceed as if they were inhibited by a salt compound formed by the union of NaCl and $CaCl_2$ with some constituent of the protoplasm (certain of these reactions are accelerated by $CaCl_2$).

3. In this and preceding papers a quantitative theory is developed in order to explain: (*a*) the toxicity of NaCl and $CaCl_2$; (*b*) the antagonism between these substances; (*c*) the fact that recovery (in sea water) may be partial or complete, depending on the length of exposure to the toxic solution.

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THE RATE OF OVULATION IN THE DOMESTIC FOWL DURING THE PULLET YEAR.

By SAMUEL BRODY.

(*From the Missouri Agricultural Station, Columbia.*)

(Received for publication, January 18, 1921.)

It is well known among poultrymen that ovulation in the hen is an orderly process with a fairly predictable average production of eggs for each month of the year. The object of this paper is to contribute an idea toward the formulation of an hypothesis concerning the principles underlying this orderly process of ovulation. This idea is based on a suggestion of Loeb[1] that growth, or at any rate the limiting factor of growth, is in the nature of an autocatalytic monomolecular reaction. The rate of ovulation may reasonably be taken as an index of growth of the eggs, and if the limiting factor of growth of eggs is an autocatalytic reaction, then the rate of ovulation should be expressible by the equation of such a reaction.

The application of this equation to experimental data is familiar to the chemist.[2] Robertson[3] and Ostwald[4] were the first to apply the equation to the phenomena of growth. In the formulation of the equation it is assumed that the velocity of reaction at any moment is proportional to the quantity of the reacting substance $(A - x)$ at that moment, and also to the quantity of the product of the reaction

[1] Loeb, J., *Biochem. Z.*, 1907, ii, 34; *7th Internat. Zool. Cong.*, 1907; The dynamics of living matter, New York, 1906; *Univ. California Pub., Physiol.*, 1905–10, iii, 61.

[2] Ostwald, W., Lehrbuch der allgemeinen Chemie, Leipsic, 1903, i. Lewis, G. N., *Z. physikal.Chem.*, 1905, lii, 310; *Dept. Interior, Bureau Gov. Labor., Chem. Lab., No. 30*, 1905.

[3] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; *Am. J. Physiol.*, 1915, xxxvii, 1, 74; Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920.

[4] Ostwald, W., Vorträge und Aufsätze über Entwicklungsmechanik Organismen, Leipsic, 1908, v.

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x, present at that moment, which acts as the catalyst of the reaction; that is

$$\frac{dx}{dt} = K_1 x\,(A - x) \quad (1)$$

where $\frac{dx}{dt}$ stands for the momentary velocity or rate of reaction, and K_1 is the velocity constant. Equation (1) cannot be applied to experimental data since the momentary velocity of the reaction is not known, and since the velocity is not expressed as a function of time. It is therefore first integrated obtaining

$$\log \frac{x}{A - x} = K_1 A t + C \quad (2)$$

where C is the integration constant. The value of the integration constant C is found by an analysis of the meaning of t in equation (2) above. Equation (1) represents a curve of a rising and falling type, the maximum or turning point occurring when $x = A - x$; that is, when the reaction is half way completed, and when the reaction is at the maximum velocity. At that point, therefore, $\log \frac{x}{A - x} = 0$. It is most convenient to count the time from this maximum or turning point, that is t at this point is just equal to zero; therefore, $K_1 A t = 0$ and also $C = 0$; if it is agreed to count time from the maximum velocity, then $C = 0$, and equation (2) becomes

$$\log \frac{x}{A - x} = K_1 A t \quad (3)$$

t in equation (3) is then the time on either side of the maximum point, counted from that point as zero. Equation (3) may be more conveniently written

$$\log \frac{x}{A - x} = K_1 A\,(t - t_1) \quad (4)$$

where t_1 is the time from the beginning of the reaction to the maximum point; t is any time from the beginning of the reaction chosen for discussion; and $(t - t_1)$ is therefore the difference of time from the maximum point to the chosen time t. The minus sign between t and t_1 indicates difference in time between the maximum and

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any chosen time t, anywhere along the reaction curve, on either side of the maximum, rather than that t_1 is negative. Equation (4) may be still further simplified by writing it

$$\log \frac{x}{A - x} = K (t - t_1) \quad (5)$$

where K is written for K_1A. Equation (5), used by Robertson[5] in the study of growth, may now be applied to the study of data on ovulation.

A large amount of data on ovulation of the domestic fowl is found in the work of the several Agricultural Experiment stations in this country. The best known published records are undoubtedly those prepared by Pearl[6] on the weighted mean monthly egg production of barred Plymouth Rock pullets representing 4,210 birds covering the records kept from 1899 to 1907 at the Maine Agricultural Station, and the results of the international egg laying contests conducted by the Storrs Experiment Station.[6] The average monthly production of 1,000 White Leghorn pullets during the seventh international contest[6] will be taken as the second example for computation.

It might be well before applying equation (5) to these data to redefine them in terms of ovulation during the pullet year. x = number of eggs laid from November 1 of the pullet year up to the end of any month, t. A = total number of eggs laid in the *natural laying season* which lasts very nearly 1 year, November 1 of pullet year to Novem- 1 of the succeeding year. Some eggs may be, and in fact are, laid outside this arbitrarily defined limit of 1 year. However, all published records adhere to this arbitrary year, and hence A is tentatively defined as eggs laid from November 1 to November 1 of the succeeding year. t_1 = time in month when $x = A - x$ = time required to lay half of the total number of eggs laid in 1 year = time when the monthly rate of laying is at its maximum. K = velocity constant found by substituting values for x, A, t, and t_1, and solving for K. Substituting the values of A, K, t, and t_1, and solving for x, we obtain the following calculated values for the two examples chosen,

[5] Pearl, R., *U. S. Bureau Animal Industry, Bull. 110*, 1910, pt. 2.

[6] Card, L. E., and Kirkpatrick, W. F., *Storrs Agric. Exp. Station, Bull. 100*, 1919, pt. 2, 35.

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which are tabulated with the experimental values. Robertson's tables[7] are helpful in solving for x, the calculated number of eggs laid.

Month.	No. of month. t	Barred Plymouth Rock.		White Leghorn.	
		Experimental values.	Calculated values.	Experimental values.	Calculated values.
Nov.	1	4.63	10.8	6.5	7.3
Dec.	2	13.54	16.8	13.1	12.2
Jan.	3	25.25	25.2	20.2	19.9
Feb.	4	36.12	36.7	30.7	31.5
Mar.	5	52.23	50.6	48.4	47.5
Apr.	6	68.08	66.1	66.9	67.5
May.	7	82.00	81.5	89.5	89.5
June.	8	94.46	95.0	109.2	110.1
July.	9	105.53	105.8	127.2	127.5
Aug.	10	115.17	113.7	143.8	140.2
Sept.	11	123.36	119.2	156.8	149.0
Oct.	12	128.86	123.0	162.8	154.8

Similar results are obtained with the other principal breeds of birds, the equation for Plymouth Rocks being

$$\log \frac{x}{129 - x} = 0.212\ (t - 5.9)$$

and the equation for White Leghorns

$$\log \frac{x}{163 - x} = 0.235\ (t - 6.64)$$

In the equation for White Leghorns, 163 is the value of A since the total number of eggs laid in the year is in round numbers 163 eggs. It takes 6.64 months to lay half this number of eggs. 0.235 is the constant of the reaction.

It is clear from the tabulation that the agreement between the experimental and calculated values of x (eggs laid) is very good for three-quarters of the laying season—January to August. On the other hand the per cent deviation in the first 2 months (November and December) and the last 2 months (September and October) is very great. The deviations in the last 2 months (September and

[7] Robertson, T. B., *Univ. California Pub., Physiol.*, 1910–15, iv, 211.

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October) can be easily corrected by assuming the value of A to be slightly larger than the recorded number of eggs laid during the arbitrarily defined laying year of November 1 to the following November 1. Thus, instead of taking the value of A to be 163 eggs for the

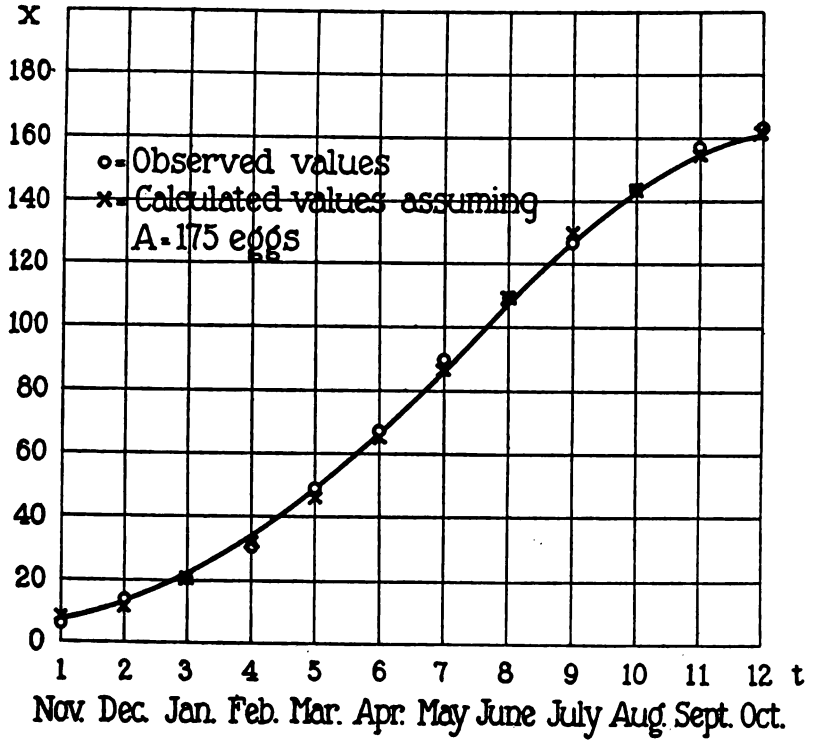

FIG. 1. Curve showing the average number of eggs laid by the White Leghorn fowl at the Storrs Seventh International Egg Laying Contest. Ordinates represent the number of eggs laid (x) for the time t; abscissæ represent the time t from Nov. 1, the beginning of the observation, to any time of the year.

White Leghorns, 175 eggs are taken, an increase of only 12 eggs over the recorded value, a number which will probably be laid by this breed outside the arbitrary, conventional year; then an excellent agreement is obtained as shown in the following tabulation and in Fig. 1.

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Assume then $A = 175$; t_1 is shifted to 7 months and the equation becomes

$$\log \frac{x}{175 - x} = 0.222\ (t - 7)$$

obtaining the following values for x for the White Leghorns.

Month.	No. of month. t	White Leghorn.	
		Experimental value.	Calculated value.
Nov.	1	6.5	7.9
Dec.	2	13.1	12.6
Jan.	3	20.2	20.1
Feb.	4	30.7	31.0
Mar.	5	48.4	46.4
Apr.	6	66.9	65.6
May.	7	89.5	87.5
June.	8	109.2	109.2
July.	9	127.2	129.0
Aug.	10	143.8	144.0
Sept.	11	156.8	155.0
Oct.	12	162.8	162.4

The agreement is seen to be excellent except during the first 2 months (November and December). The discrepancy during the first 2 months cannot, however, be considered serious, in view of the fact that the initiation of chemical processes even *in vitro* is in many cases irregular and does not follow the mathematical expressions for the rate of reaction. The initiation of processes in living organisms, especially in the case of ovulation, may be attended in addition by purely mechanical difficulties and irregularities. The discrepancy might also be explained on evolutionary grounds following the argument of Pearl.[5] Pearl calls attention to the fact that the wild *Gallus*, the ancestor of the domestic fowl, does not lay during the winter months, and that the winter laying period is not a part of the natural or normal reproductive cycle of the hen. The high variability of egg production during this period is explained by Pearl on this basis.

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SUMMARY.

The rate of ovulation of the domestic fowl may be expressed by the equation of an autocatalytic chemical reaction. This is not surprising in view of the fact that the rate of growth may also be expressed by such an equation, and that the rate of ovulation is probably an index of the growth of the eggs. This brings the phenomenon of ovulation in the hen under the general subject of growth, and substantiates the generality and the probability of the hypothesis that growth, or at any rate the limiting factor of growth, is an autocatalytic reaction.

The idea of applying the equation of growth to the rate of ovulation in the fowl suggested itself while studying the rate of growth of the dairy cow with Mr. A. C. Ragsdale, Chairman of the Department of Dairy Husbandry. I take pleasure in expressing my appreciation to Mr. Ragsdale, and Mr. H. L. Kempster, Chairman of the Department of Poultry Husbandry in this station, for their encouragement.

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THE PHAGOCYTOSIS OF SOLID PARTICLES.

I. Quartz.

By WALLACE O. FENN.

(From the Laboratory of Applied Physiology, Harvard Medical School, Boston.)

(Received for publication, January 15, 1921.)

Measurement of Phagocytosis.

The usual procedure for measuring phagocytosis is to incubate suspensions of leucocytes and solid particles or bacteria together in a test-tube for a given length of time; then to remove a sample and make a smear of the mixture on a slide which is stained, mounted, and counted at leisure. When bacteria are used as objects for ingestion, as in opsonic index determinations, the actual number of bacteria inside a given number of leucocytes is counted and comparisons are made on the basis of the average number of bacteria taken up per leucocyte per unit of time. It is impossible to do this with solid particles which are usually larger than bacteria and hence may almost completely fill the cell in a short time or become easily superimposed.

For this reason Hamburger (1), the author of the only extensive quantitative experiments on phagocytosis of solid particles, always counted the per cent of leucocytes containing solid particles per unit of time. On theoretical grounds this method possesses the objection pointed out by McKendrick (2) that it is not a measure of the amount of work done but a measure of the number of cells which have done the work. He shows, however, that if a normal frequency curve for the distribution of the bacteria in the leucocytes is assumed, the number of bacteria per leucocyte, *i.e.* the amount of work done, can be calculated from the per cent of empty leucocytes, the former being a logarithmic function of the latter, and he suggests that the determination of opsonic index may be simplified by counting only the empty leucocytes.

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Madsen and Watabiki (3) have avoided this difficulty, in measurements of the rate of phagocytosis of bacteria at different temperatures, by measuring the time curves of the number of bacteria taken up per leucocyte at each temperature. By certain assumptions they succeeded in fitting their experimental curves to the formulas for monomolecular or bimolecular reactions, and thus determining a constant, K, which represented the rate of the reaction. There seemed to be in this case, however, no particular significance to the obedience of the curves to the laws for chemical reactions.

In the experiments on phagocytosis of solid particles to be described in this paper it was found possible to avoid the objections in former methods and to analyze the reactions quantitatively in terms of the number of collisions occurring between cells and particles. To accomplish this, particles of carbon or quartz of uniform size were incubated with leucocytes. At frequent intervals a sample was removed to an ordinary blood-counting chamber and the *number of particles not taken up* by the leucocytes was counted.

If the number of cells present is large and remains constant throughout the experiment, the number of collisions varies only with the number of particles, and it should be possible to calculate the rate of phagocytosis, K, from the equation for a monomolecular reaction, $K = \frac{1}{t} \log \frac{A}{A - x}$, where A is the number of particles originally present and x is the number ingested by the leucocytes in the time, t. The fact that K is usually found to be constant shows that we are dealing with a process like a monomolecular reaction in which the collisions can be watched under the microscope. The leucocytes can take up so many particles that their capacity does not diminish sufficiently to limit the rate of the reaction. Certain uncontrollable complicating factors which cause K to vary will be discussed with the experimental results and in a subsequent paper.

A constant K, calculated in this way, means that the same per cent of the particles present is being ingested per unit of time; *i.e.*, K is independent of the actual number of particles present. In other words, it is *not the number of collisions but the chances of collision which determine* K, other things being equal. Since the chances of collision

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depend upon the size and density of the particles in relation to the size and density of the cells, large particles should be taken up more rapidly than small particles. This was found to be true, and, moreover, a direct proportionality was found to exist between the experimental constant, K, and the relative chance of collisions as calculated. It will be seen that this method presents great difficulties as a means of comparing the rate of phagocytosis of different particles and it was finally discarded in favor of a simpler method which will be described in a subsequent paper. The results by the first method possess considerable theoretical interest, however, and the consideration of the chances of collision should be of interest perhaps in opsonic index work where this factor has never been taken into consideration.

The technique of handling the quartz suspensions will first be described; then the method of calculating the chances of collision; and the experimental results, using three different sizes of quartz particles. Similar experiments with carbon and comparisons between quartz and carbon will be described in later papers.

The Suspensions.

The first requirement for these experiments is to have the particles of as nearly uniform size as possible. Suspensions prepared by Hamburger's method would not be sufficiently uniform to admit of a calculation of the chances of collision. Uniformity can be obtained by centrifugalization or settling. The former is quicker but the product is not so good nor so easily controlled. In both methods the principle consists in washing out the particles which are too small. The larger particles may be left in the suspension until a uniform sample is needed, when the suspension is shaken up and the large ones are either centrifugalized out or allowed to settle out by gravity. In the centrifuge, however, it is quite impossible not to set up currents which carry some excessively large particles to the top. The small particles can, however, be removed in the centrifuge and the sediment can thus be resuspended and allowed to settle out by gravity. It settles with a sharp line at the top from which a uniform sample can be withdrawn.

This combined method was the one usually used in these experiments. Suspensions of this sort of carbon and quartz have been

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kept for nearly a year at room temperature, without sterilizing, and there has always been an abundant supply of uniform particles in each when shaken up and allowed to settle. *Uniform* suspensions cannot be kept, however, without the addition of acacia or some other protective colloid to prevent agglutination.

Chances of Collision.

If a suspension of cells of diameter C and velocity (under the influence of gravity) Vc, and of particles of diameter P and velocity Vp, is allowed to settle in a test-tube, the chances of collision, R, between them will be proportional to the velocity of the particle relative to the leucocyte and to the square of the sum of their diameters or

$$R = (Vp - Vc)\ (C + P)^2 \qquad (1)$$

The last factor is derived by a consideration of Fig. 1. If an infinitely small particle is settling down, its chance of hitting a single cell C is proportional to the cross-sectional area of C or πC^2. If, however, the particle has a finite diameter P, its center may miss the edge of the cell by a distance $\frac{P}{2}$ and still collide. The effective cross-sectional area of C as a target for a particle is thus increased to $\pi(C + P)^2$. The chance that P will hit C is, therefore, proportional to $(C + P)^2$.[1]

In these experiments, however, the suspensions were not allowed to settle out in stationary test-tubes as the formula could no longer be applied to cells and particles which were resting on the bottom. Instead, the test-tubes holding 1 to 2 cc. of the mixture were placed horizontally on a drum revolving slowly about a horizontal axis. It can be shown, however, that the same formula applies to this case. Consider first a case where there is no air bubble in the tube. Each cell and particle in the mixture is settling at a constant rate in a uniformly rotating medium, and at the end of one revolution will have returned exactly to the original position in the tube, having

[1] The writer is indebted to Dr. E. K. Carver, National Research Fellow in Chemistry, of the Wolcott Gibbs Memorial Laboratory of Harvard University, for assistance with this formula.

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described a circle the circumference of which is equal to the distance which each cell or particle would have settled during the time of one revolution either in a straight line or otherwise.

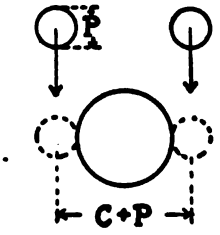

FIG. 1. Diagram illustrating chance of collision between a particle, P, settling down toward a cell, C.

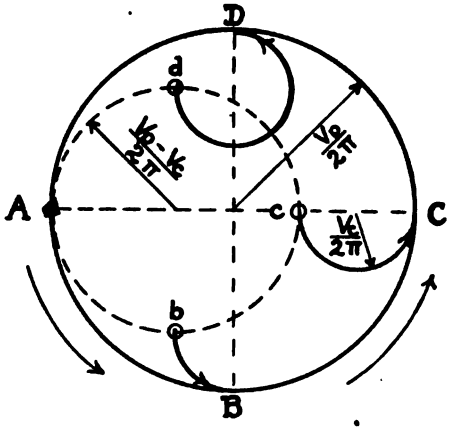

FIG. 2. Diagram illustrating orbits described by a particle at A and cells at b, c, and d in a rotating medium. The figure represents a cross-section of the medium which is considered to be rotating clockwise. The relative direction of rotation of particles and cells settling by gravity is, therefore, counter-clockwise, as indicated by the arrows. Collisions occur at B, C, and D. Chance of collision is proportional to the circumference, $Abcd$.

The circumference thus equals $\frac{V}{n}$, where n is the number of revolutions per unit of time and V the velocity. Let the circle $ABCD$ (Fig. 2) be the orbit described by a particle, P, starting at A in the direction

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of the arrow. Its radius equals $\frac{Vp}{2\pi}$.[2] Then the cells of velocity Vc which will collide with the particle at B, C, and D after one-quarter, one-half, and three-quarters of a revolution, respectively, must have been originally located at the points b, c, and d. But all these points lie on a circle (Fig. 2) whose radius is $\frac{Vp}{2\pi} - \frac{Vc}{2\pi}$, and whose circumference is therefore, $Vp - Vc$. Obviously the chance of collision depends upon the length of the line upon which colliding cells may lie.

In some of the following experiments, however, these ideal conditions were not quite realized, especially at the beginning before the theory of the chances of collision had been thoroughly worked out. Instead, an air bubble was allowed to be present and in many cases traveled from one end of the tube to the other as it revolved, owing to the fact that the tube was not quite horizontal. As the bubble passes along the tube it leaves behind it little eddies. These may be seen in a thick suspension of quartz as layers of unequal concentration of particles caused by the piling up of particles in the eddies by centrifugal force. It was observed that counts of the number of particles from small amounts of samples (5 c.mm.), removed immediately after stirring, showed greater variations than from samples taken when these inequalities in concentration had disappeared. It is probable that both particles and cells are acted upon by centrifugal force when stirred up, and move, therefore, with exactly the same *relative* velocities as when settling under the force of gravity alone. However the mixture is stirred, there would be no collisions if all particles and cells had exactly the same velocities, for they would merely be carried along by the current. Any collisions, then, must be due to differences in velocities.

In the smallest of these particles brownian motion was just perceptible. Even if the particles were so small, however, as to have no velocity under gravity and therefore very active brownian motion, this fact would not affect the calculation of the chances of collision with a cell which is literally "sweeping up" the particles with a velocity

[2] n is taken equal to 1.

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of 1.67 cm. per hour, since the number of particles which got in front of the cell by their brownian motion would be just balanced by the number which would get out of the way of the cell.

Verification of the Theory by Varying the Speed of Rotation.

According to the theory, then, the chances of collision determine K, other things being equal. The fact that the chance of collision is proportional to $Vp - Vc$, *i.e.* the difference between the speeds of settling of particles and cells, makes possible a verification of the theory by varying the speed of rotation of the tubes in which the phagocytic mixtures are incubated.

Returning to Fig. 2, it is evident that the orbits described by the particles, being equal to $\frac{V}{n}$, must decrease with an increase in n, the number of revolutions per unit of time. It should be possible by increasing n to decrease the diameter of the orbits nearly to zero. Under such conditions there should be less phagocytosis than when the mixture is rotated more slowly. In a slowly rotated mixture the particles have plenty of time to settle down to the neighboring cells. To test this prediction under ideal conditions, six small tubes, 3 mm. inside diameter and 1.5 cm. long, sealed at one end, were prepared. At the beginning of the experiment all these tubes were filled with the mixture of cells and quartz particles immediately after the cells were added. The open ends of the tubes were then sealed by dipping in melted paraffin, excluding all air.

Three of the tubes were then put on a drum rotating at 0.3 revolution per minute and three on one rotating at 19 revolutions per minute. Counts were then made of the number of particles present in the remainder of the original mixture. At intervals of about 1 hour one tube was taken from each drum and the number of particles not yet ingested was counted. From these data the phagocytosis constant, K, was calculated by the equation for a monomolecular reaction.

As predicted, the particles are ingested nearly two and one-half times as rapidly in the slowly revolving tube. Likewise, the cells aggregate more rapidly in the slow tube, due to the greater number of collisions between them.

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The result was as follows:

Hrs.	19 revolutions per minute.		0.3 revolution per minute.	
	No. of particles.	No. of cells.	No. of particles.	No. of cells.
0	324	76	324	76
0.6	262	76	124	48
1.75	157		55	
2.6	64	53	9	32
	$K = 0.22$		$K = 0.52$	

(Counts refer to volumes of 0.02 mm.^{3})

The diameter of the orbits of cells and particles has been calculated from the equation Diameter $= \frac{V}{\pi n}$, and is compared in the following tabulation with the average distance between cells and particles calculated from the equation

$$\text{Average distance} = \sqrt[3]{\frac{\text{Total volume of suspension}}{\text{No. of cells} + \text{No. of particles}}} = 37\mu$$

	Cells. μ	Particles. μ
Average diameter	9.0	2.9
Diameter of orbit at 19 revolutions per min.....	4.6	7.3
" " " " 0.3 revolution " "	279	462

These results show that the diameters of the orbits of cells and particles at 19 revolutions per minute are small compared to the distances between them. It is, therefore, only due to the fact that the laws of chance prevent all particles from being equidistant from each other (*i.e.* 37μ) that there was any phagocytosis at all at 19 revolutions per minute. In this case, also, when the cells are practically stationary, brownian motion probably plays some part in bringing particles to the cells. Centrifugal force is another factor which would have a very slight effect in causing collisions. It was calculated that at 19 revolutions per minute 12 per cent of the particles and 8 per cent of the cells would be centrifugalized against the wall of the tube and that it would take 21 minutes for a particle to overtake a cell 37μ distant,

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both moving under centrifugal force alone. Centrifugal force is not, therefore, an appreciable factor except in the case of those cells which become "plastered" against the wall of the tube and there ingest particles. This could have been avoided by rearranging the tubes on the drum at intervals.

This experiment was done under ideal conditions, however, in that there was no air bubble present in the tube. When such a bubble is included in a tube which is slightly inclined to the horizontal, it travels up and down in the tube and stirs the mixture. Under such conditions the rate of phagocytosis, K, should vary in direct proportion to the speed of rotation. This also is true. Thus K was increased from 0.17 to 0.76 (4.5 times) by an increase from 7 to 27 revolutions per minute (3.9 times).

These two experiments offered a very satisfactory proof of the theory at the outset and the following predictions may be made with considerable assurance.

1. The rate of phagocytosis, K, may be calculated from the equation for a monomolecular reaction and will remain constant in any experiment as long as the concentration of cells remains constant.

2. For a given leucocyte suspension in a given medium, K is determined by the chances of collision between cells and particles.

3. The chances of collision vary with the method of stirring (*i.e.* speed of rotation, etc.) but the *relative* chances of collision, R, are applicable however the cells and particles are kept in suspension.

4. The relative chance of collision of any particle is given by the formula $R = (C + P)^2 (Vp - Vc)$.

5. The equation $\frac{K_1}{K_2} = \frac{R_1}{R_2}$ should be true for any two sizes of particles, 1 and 2.

Velocity of Particles.

The relative chances of collision are, therefore, very simply calculated if the diameter and velocity of the particles and cells are known. The simplest way of determining the velocity of the particles is by direct observation of the speed with which the suspensions settle out in the centrifuge bottle. This can only be done accurately in a suspension of particles of such a uniform size that they all settle at

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the same rate leaving a clear liquid above. In all the suspensions used in these experiments this was possible. From the velocity so obtained, the diameter can be calculated by Stokes's law.

For example, if a suspension has been allowed to settle for 1 hour until 5 cm. of clear water remain at the top, the upturned capillary tip of a siphon is now placed 7 cm. below the surface, and the upper 2 cm. of the suspension are drawn off, the velocity of these particles is somewhere between 5 and 7 cm. per hour. It is probably given most accurately by the formula

$$V_{av} = V_g - \frac{V_g - V_s}{\sqrt{2}} \qquad (2)$$

where V_s and V_g are the smallest and largest velocities, respectively. This may be seen from a consideration of Fig. 3. Here the abscissæ represent velocities. Ordinates represent the number of particles of

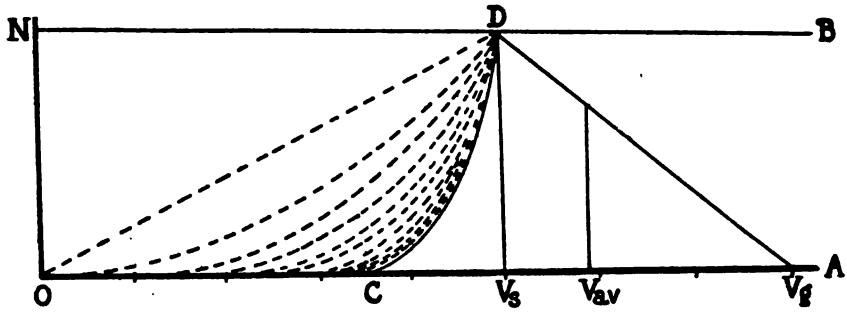

FIG. 3. Ordinates represent number of particles, abscissæ represent velocities or diameters squared. Original raw material is represented by *AONB*, *N* particles of each velocity being assumed. Dotted lines show portions removed after ten successive settlings. *ACDB* is the permanent suspension before large particles are removed. V_gCD is, then, the frequency curve of a uniform suspension removed from the top as suspension *ACDB* settles. V_{av} is the average velocity.

each velocity originally present; it is assumed that there were the same number, *N*, of each in the original raw material. The area, *AONB*, then represents the original suspension. The dotted lines *DO*, etc., outline the parts of the suspension which were successively discarded with the supernatant liquid after being allowed to settle ten times until particles of velocity, V_s, had just settled out. 50

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per cent of the particles, which settle only half this distance, are removed each time.

The suspension as kept permanently is then represented by the area $ACDB$. To prepare a uniform suspension the whole is then shaken up, allowed to settle, and the upper layer of the suspension pipetted off at such a point that only particles of velocity smaller than V_g are obtained. The final suspension is then represented by the area V_gCD, and the average velocity must be measured by the perpendicular bisector of this area. Since V_sCD is small compared to the whole it may be neglected, particularly as V_s is necessarily more or less indeterminate in practice. The average V is, then, best given by the bisector of the triangle V_gV_sD or V_{av} which can be shown by elementary geometry to have the value given in equation (2) above.

Stokes's law (4) gives the velocity, V, in centimeters per second of a spherical body of radius, r, and density, D, in a medium of absolute viscosity, η, and density, d, as

$$V = \frac{2(D-d)}{9\eta} gr^2$$

whence

$$r = \sqrt{\frac{9V\eta}{2g(D-d)}}$$

Introducing values $\frac{0.010 \text{ erg second}}{\text{centimeter}^3}$ for η (5) and 981 $\frac{\text{centimeter}}{\text{second}^2}$ for g, and changing V into velocity in $\frac{\text{centimeter}}{\text{hour}}$ and r from centimeters to microns we have

$$r = \sqrt{\frac{4.5\ V\ 0.01 \times 10^8}{981\ (D-d)\ 3{,}600}}$$

and

$$\text{Diameter} = \sqrt{\frac{5.1\ V}{(D-d)}} \quad (3)$$

For the purposes of these experiments it is possible to use this formula directly without allowing for the differences in the density and viscosity of the phagocytic mixtures. The viscosity has no effect on the relative chances of collision because it modifies the

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velocity of both the cells and particles equally. Since the density of the medium was only 1.01 this can also be disregarded as small in comparison with the density of the particles, 1.81 (carbon) and 2.68 (quartz). As far as the leucocytes were concerned with a density 1.11, this was really allowed for largely by the necessity of measuring their velocity in a medium of sodium chloride of density 1.007.

In the case of suspensions which are not uniform enough to settle with a sharp boundary[3] it is impossible to determine anything but the velocity of their smallest particles by direct observation. For such cases a method was devised which is known as the "stop-cock" method. It was also necessary to use this method for determining the velocity of the leucocytes. The method consists in allowing the suspension to settle out in a glass tube provided with a specially made stop-cock, such that there was no constriction in the inside diameter; there was, thus, a straight uniform tube all the way through the stop-cock. After a given interval of time the stop-cock is turned. The change of concentration in the suspension above the stop-cock is determined by counts of the numbers of particles present before and after settling. If there are h centimeters of suspension above the stop-cock and if the concentration before and after is given by C and C_1, the velocity is given by the formula

$$V = \frac{h\,(C - C_1)}{Ct} \qquad (4)$$

This method gives an average figure for the velocity because the small particles which settle too slowly compensate for the large ones, all of which may have passed below the stop-cock before it is turned. Obviously, however, the diameter which is calculated from the average velocity is not the average diameter because the velocity is proportional to the square of the diameter. The agreement between results by this method and measurements of the diameter of particles by microscopic methods was, however, sufficiently good for these purposes as shown in Table I. The agreement is in itself proof of the uniformity of the suspensions used.

In the table are also included some figures for the diameters of these suspensions by the method of evaporating to dryness a suspension containing a known number of particles, and calculating the

[3] This is the case with suspensions from which the large particles were removed by centrifuge instead of by settling.

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diameters on the assumption that each particle is a sphere. It is doubtless due to the irregular sizes of the particles that this method gives results which are uniformly lower than the other methods. At the end of the table are also included some figures for the smallest diameters of the particles as calculated from the velocity with which the upper boundary of the suspension settles under gravity; *i.e.*, the velocity of the smallest particles. This figure can readily be obtained from the stop-cock method in addition to the figure for the

TABLE I.

Measurements of Diameters in Microns of Three Sizes of Quartz Particles by Various Methods.

Method.		Diameters.		
		Size 1.	Size 2.	Size 3.
		μ	μ	μ
Dry weight		3.55	3.2	
		3.46	3.14	2.16
Microscopic		5.01	4.34	2.32, 2.44
		4.63 ± 0.05	4.08 ± 0.05	2.93 ± 0.036
Stop-cock method	Counting	4.67	4.44	2.82
		4.98	4.10	2.62
	Colorimeter	5.05 ± 0.14	4.05 ± 0.08	2.7 ± 0.04
Smallest diameter		3.04	2.65	1.93
		3.26		2.16

average velocity and gives a good idea of the degree of uniformity of the suspension. To avoid counting, the stop-cock method was usually modified by the use of the colorimeter for comparing the concentration of the suspension before and after settling.[4] All the measurements in Table I were made on suspensions prepared separately

[4] For this purpose underneath illumination only was used so that the intensity of the transmitted, not the diffracted, beam was measured. The use of the colorimeter is perhaps a doubtful measure on theoretical grounds unless the suspensions are thoroughly uniform, because it assumes that the amount of light transmitted is proportional to the number of particles without respect to size. After settling there are of course relatively fewer large particles.

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from the same original stock bottle, the large particles being removed by centrifugalization each time. It is not surprising, therefore, that the measurements should differ somewhat at different times even by the same method.

Diameter and Velocity of Leucocytes.

The leucocytes were obtained from rats. The animals were injected intraperitoneally with a suspension of aleuronat, and the peritoneal cavity was opened on the following day and washed out with 0.5 per cent sodium citrate in 0.9 per cent sodium chloride solution. The cells were then centrifuged once to get rid of the citrate and resuspended in salt solution. If the cells are not washed thus it is necessary to have so much citrate present to prevent clotting of the exudate that it prevents phagocytosis.

For these experiments it was of first importance to determine the diameter and velocity of the leucocytes. These determinations had to be done independently as the density could not be measured directly. The diameter was measured directly in the microscope on fresh specimens before they had had time to spread on the microscope slide. The velocity was determined by the stop-cock method. An average of five determinations was 1.67 ± 0.05 cm. per hour. The figures were as follows: 1.63, 1.56, 1.83, 1.47, 1.84.

Since, however, the leucocytes are not of the same size it is necessary, in order to calculate the chances of collision with any accuracy, to divide the cells into three groups of three average diameters and calculate the chances of collision for each group separately. These calculations were made as follows: The diameters, D, of the leucocytes were measured as already described. From these data a frequency curve was obtained which was divided into three parts (Table II). The relative velocities of the three groups were then calculated by the formula $V = D^2K$, where K is a constant. From these velocities the average velocity may be calculated as shown by multiplying the velocity for each group by the percentage of total cells in that group and dividing the sum of the results by 100. But the average velocity was found by experiment by the stop-cock method to be 1.67 ± 0.05. Equating these we have

$$1.67 \pm 0.05 = 81.0\,K$$
$$K = 0.0206 \pm 0.00062$$

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Substituting this value for K in the third column of Table II we have the true values for the velocity in the last column. These were the data used in calculating the chances of collision.

TABLE II.

Diameters and Velocities of the Three Groups of Leucocytes.

Cell group.	Average diameter.	Relative velocity × per cent of total cells.	True velocity.
	μ		*cm. per hr.*
1	7.6 ± 0.04	$58.0\,K \times 0.32 = 18.6\,K$	1.20 ± 0.035
2	8.9 ± 0.016	$79.5\,K \times 0.39 = 31.0\,K$	1.64 ± 0.048
3	10.8 ± 0.034	$108.5\,K \times 0.29 = 31.4\,K$	2.24 ± 0.066
Average velocity.....		$81.0\,K$	1.67 ± 0.05

From these data the density of the cells may be determined by equation (3).

$$D - d = \frac{5.1 \times 1.64}{8.9^2} = 0.105$$

$$D = 1.007 + 0.105 = 1.11$$

Comparison of the Rates of Phagocytosis of Quartz Particles of Three Different Sizes.

Experiments 1 and 2.—The three quartz suspensions were washed once with distilled water and were then allowed to settle at room temperature. After the sharp boundary of the suspension had settled 3 or 4 cm. from the top of the liquid, a definite amount, about 2 cm., was siphoned off the top of each suspension as already described. From the minimum and maximum velocities so obtained, the average velocity is calculated by equation (2) (Table III). The diameter is then given by equation (3), and the chances of collision with the leucocytes by the formula

$$R_{\text{total}} = \frac{R_a P_a + R_b P_b + R_c P_c}{100} \qquad (5)$$

where R_a, R_b, and R_c are the chances of collision with the three groups of cells a, b, and c; and P_a, P_b, and P_c, the percentages of cells in these groups. The velocity of the 2.55μ particles being most nearly equal to that of the cells, these particles have the smallest chance of collision, as shown in Table III. Under the microscope these suspensions appeared as absolutely uniform as any suspension of irregular particles could be (Fig. 4).

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The phagocytic mixtures were prepared as follows: 0.2 cc. of washed leucocyte suspension in 0.9 per cent sodium chloride, plus 0.15 cc. of quartz suspension in 0.9 per cent acacia plus 0.1 cc. of fresh serum plus 0.05 cc. of M/10 phosphate mixture ($[H]^+ = 3 \times 10^{-8}$) plus 0.05 cc. of 4.5 per cent sodium chloride. The acacia was added to prevent agglutination of the quartz. The phosphate helped to maintain the hydrogen ion concentration equal to that of blood serum. Frequent colorimetric measurements of $[H]^+$ at the close of the experiments showed no appreciable change in reaction from the original. Without serum there is very little phagocytosis.

TABLE III.

Comparison of Theoretical and Experimental Rates of Phagocytosis of Three Sizes of Quartz Particles.

Average diameter of particles.			2.92 μ	2.55 μ	1.85 μ	Ratio.
Velocity per hr. in cm.	Minimum		2.51	1.89	1.00	
	Maximum		3.54	2.73	1.44	
	Average		2.81	2.14	1.13	
Chances of collision			151.8	61.8	76.5	1:0.41:0.51 (Theoretical.)
Experiment 1 (Fig. 5)	K		0.078	0.033	0.05	1:0.42:0.64 (Experimental.)
	Average per cent of error	Assumed..	9.0	4.0	7.0	
		Expected.	7.0	5.0	5.7	
Experiment 2 (Fig. 6)	K		0.38	0.33	0.22	1:0.87:0.58 (Experimental.)
	Average per cent of error	Assumed..	7.0	3.1	4.4	
		Expected.	8.0	7.6	6.7	
						1:0.64:0.61 (Average experimental.)

The above mixture was incubated at 37°C. in small glass-stoppered vials 6 mm. in outside diameter, which were rotated about their horizontal axis once in 2 minutes. The speed of rotation was kept constant in any one experiment. Since the tubes were so small and were held horizontal, there was very little stirring by the air bubble included at one end of the tube. Counts of the number of particles outside the phagocytes per 0.02 mm.^{3} of solution were made at intervals. The logarithms of these counts were then plotted as ordinates against time as abscissæ. If phagocytosis follows the laws of monomolecular reactions these points should lie on a straight line of slope K, where K is the velocity of the reaction. A summary of Experiments 1 and 2 is given in Table III and the experimental points are plotted in Figs. 5 and 6.

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In Experiment 1, K and logarithm A were determined by the method of least squares; in Experiment 2, they were measured graphically. In the latter instance K was nearly ten times as high as in the former. This may have been due to a difference in the cells or in the serum. The quartz suspensions used were identical.

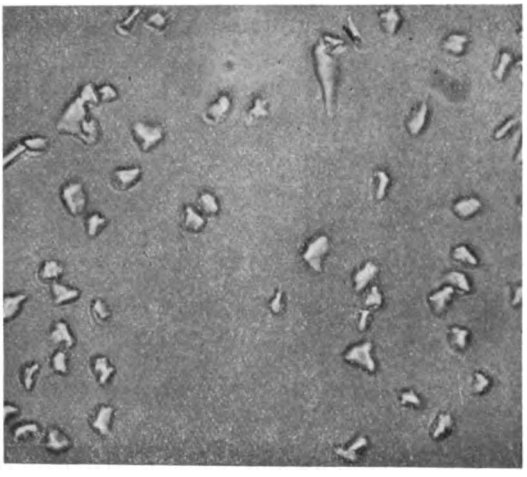

FIG. 4. Photograph of quartz suspension 4.6 μ in diameter, to show uniformity in size of particles.

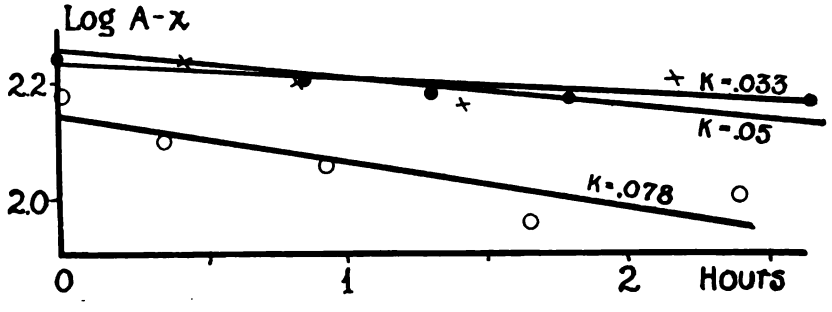

FIG. 5. Experiment 1. Ordinates represent logarithms of the number of particles not taken up by leucocytes plotted against time as abscissæ. K is equal to the slope of the graph. Quartz particles of three different sizes are compared, designated as in Fig. 6. Experimental ratios, 1:0.42:0.64; theoretical ratios, 1:0.41:0.51. K determined in this experiment by method of least squares. See Table III.

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In Table III is also given the average per cent of error which was assumed in the experimental points in order to fit them to the equation and the average probable error involved in counting the particles for the experimental points. The latter value was calculated by the expression $\frac{1.05}{\sqrt{N}}$, where N was the number of particles counted.[5] The assumption that the points lie on a straight line is justified by

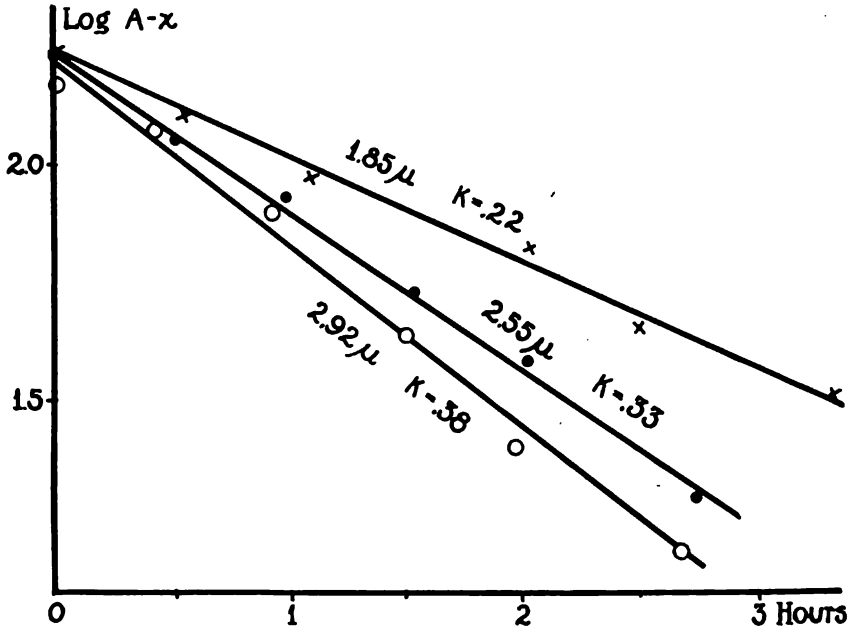

FIG. 6. Experiment 2. Legend as in Fig. 5. Experimental ratios, 1:0.87:0.58; theoretical ratios, 1:0.41:0.51. K's determined graphically. See Table III.

[5] The error of a set of observations varies inversely as the square root of the number of observations (Tuttle, L., The theory of measurements, Philadelphia, 1916, 219). The constant 1.05 ± 0.049 was determined for counting particles from calculations of the dispersion of extensive counts made on fourteen different suspensions. The dispersion of the fourteen determinations was ±0.19. The constant was also determined from counts made on forty-eight different leucocyte suspensions and gave 1.07 ± 0.05, the dispersion of the individual determinations being ±0.34.

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the fact that the error assumed is in general no larger than the probable error. Reference to Table III will show that the ratios between the three experimental constants agree fairly well with the theoretical ratios.[6]

Experiments 3 and 4.—Two more experiments were tried in which only the largest and smallest particles were compared. New suspensions were prepared from the same stock and in the same way as before. The sizes are accordingly somewhat different. The rates of phagocytosis are compared with the chances of collision in Table IV, and the experimental points are plotted in Figs. 7 and 8.

TABLE IV.

Comparison of Theoretical and Experimental Rates of Phagocytosis of Two Sizes of Quartz Particles.

Average diameter of particles.		3.18 μ	1.73 μ	Ratios.
Velocity per hr. in cm.	Maximum.....	3.95	1.91	
	Minimum.....	3.07	1.21	
	Average.....	3.33	1.41	
Chances of collision.....		286	51	1:0.18 (Theoretical.)
Experiment 3 (Fig. 7)	Initial K.....	0.133	0.046	1:0.35 (Experimental.)
	Later K.....	0.84	0.174	1:0.21 (")
Experiment 4 (Fig. 8)	Initial K.....	0.265	0.1	1:0.38 (")

In Experiment 3 the reaction starts very slowly and for some unknown reason the cells become changed or the medium becomes more favorable so that they can ingest particles more readily. Each reaction is, therefore, made up apparently of two distinct parts. If the change in the cells in the two tubes is comparable the ratio of the K's should be the same for the later K's as for the initial K's. This turns out to be true and both ratios agree well with the theoretical ratio. The inclusion of the later K's is admittedly somewhat arbitrary and is given merely for what it is worth.

The graphs in Fig. 7 for the 3.18 μ particles show considerable irregularities but the agreement of the initial K's is very good and the ratio of the initial K's for the two sizes of particles agrees fairly well with the theoretical.

[6] A discussion of the significance of a constant K in view of the gradual agglutination of cells is postponed to the next paper of this series.

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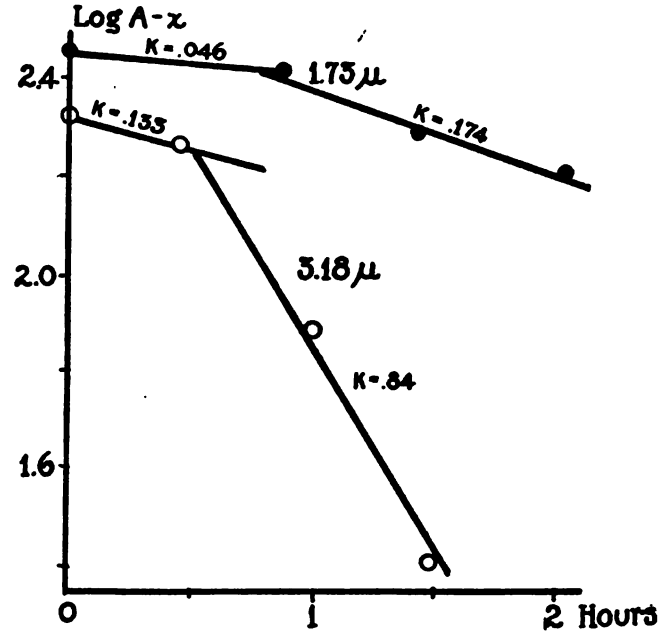

FIG. 7. Experiment 3. Ordinates represent logarithms of numbers of particles not ingested. Since K is not constant the initial K's only can be used in comparisons. Experimental ratio, 1: 0.35; theoretical ratio, 1: 0.18. Ratio of K from the end of the experiment is 1: 0.21. The two sizes of quartz particles correspond to the largest and smallest in Figs. 5 and 6. See Table IV.

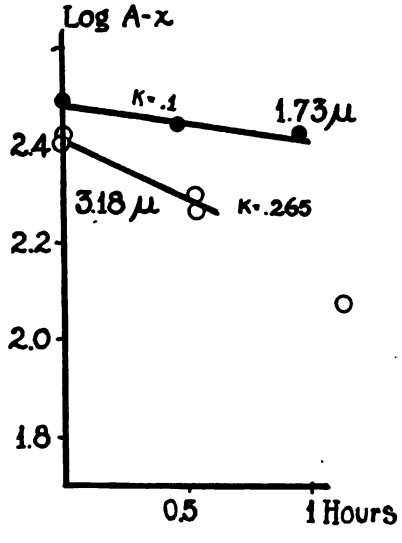

FIG. 8. Experiment 4. Repetition of Experiment 3. Only the initial K's give any basis for comparison. Experimental ratio, 1: 0.38; theoretical ratio, 1: 0.18. See Table IV.

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Experiments 5 and 6.—In two more experiments on quartz particles the suspensions used were less uniform because the large particles were removed by centrifugalization. The diameters of these particles were obtained by direct microscopic measurement. Frequency curves so obtained are plotted in Fig. 9. Each frequency curve was then divided into a number of groups and the chances of collision calculated between each one of the quartz groups and the three groups of cells by equation (5). The chances of collision for any one suspension is, therefore, equal to the sum of the products of the chances of collision of each quartz group by the per cent of particles in that group, or

$$R_{\text{total}} = \frac{R_{a_1}P_aP_1 + R_{a_2}P_aP_2 + R_{a_3}P_aP_3 + \ldots + R_{c_3}P_cP_3}{100}$$

where P is the per cent of cells or particles in the designated group, R the chance of collision; the subscripts 1, 2, and 3 refer to groups of particles while a, b, and c refer to groups of cells.

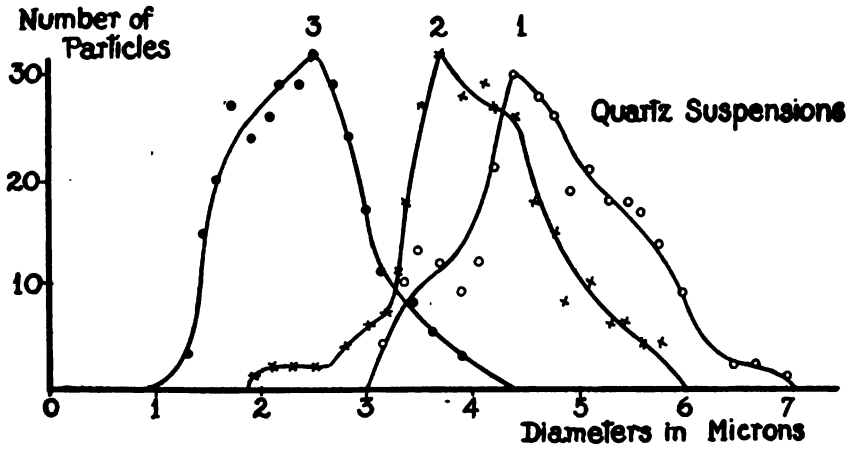

FIG. 9. Frequency curves where ordinates represent number of particles and abscissæ represent diameters of particles in microns. Data from microscopic measurements of three quartz suspensions used in Experiments 5 and 6. Average diameters equal 2.44 μ, 4.08 μ, and 4.63 μ. Points plotted are experimental points smoothed by averaging each ordinate with the two adjacent ordinates. Plotted to such a scale that the areas subtended by each graph are equal. See Table V.

The calculation in this case is much more laborious and the results are not so satisfactory. Moreover, in these experiments, which were the first comparisons attempted, larger test-tubes (8 mm. inside

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diameter) were used for incubation, and there was more irregular stirring by the air bubble which moved up and down the rotating tube. Under these circumstances the explanation offered for the different rates of ingestion of the three suspensions seems satisfactory.

The data used in calculating R are given in Table V. The velocities obtained for each group were the weighted averages of all the separate velocities in the group, not the velocity corresponding to the average diameter. The group diameters of the two larger suspensions are the

TABLE V.

Chances of Collision of Leucocytes with Three Sizes of Quartz Particles as Prepared in Experiments 5 and 6.

Average diameter of suspension.	No. of group.	Group diameter.	Group velocity.	Particles in group.	Group chances of collision.	Total R.
μ		μ	*cm. per hr.*	*per cent*		
	1	1.60	0.84	18.8	106	
	2	2.04	1.37	23.4	59	
2.44	3	2.42	1.93	19.4	54	130
	4	2.83	2.64	24.8	125	
	5	3.68	4.46	13.3	413	
	1	2.82	2.69	11.6	132	
4.08	2	4.05	5.23	71.5	602	697
	3	5.28	9.00	16.8	1,500	
	1	3.70	4.38	19.0	431	
	2	4.67	6.90	44.0	979	
4.63	3	5.45	10.00	32.0	1,745	1,299
	4	7.40	18.5	5.0	4,560	

average diameters of the group. For the smaller suspension they are calculated from the average velocity. The difference in the result is too small to have any appreciable effect. The latter method is more accurate because it is the square of the diameters which enters into the equation. The error in calculating must be larger for the smallest suspension because in this case the group velocities nearly coincide with the velocities of the groups of cells. The true chance of collision is, therefore, somewhat higher than the calculated figure. This could be avoided by the use of calculus, treating the frequency

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curves in Fig. 8 as normal frequency curves, but the resulting expression would be too difficult to work with.[7]

Since the group chances of collision are not all equal in the same suspension the chances of collision as a whole will tend to decrease as those particles with the greatest R are taken up first. It can be shown that R decreases most rapidly for the 4.63 μ particles. The ratios, therefore, increase. This ratio has been calculated for the times in the experiment when the 4.63 μ particles are 50, 75, and 90 per cent ingested (Table VI).

TABLE VI.

Comparison of Theoretical and Experimental Rates of Phagocytosis of Three Sizes of Quartz Particles.

Average diameter of particles.		4.63 μ	4.08 μ	2.44 μ	Ratios.
					(Theoretical.)
Per cent of particles ingested.....	0				1:0.54:0.10
	50				1:0.62:0.13
	75				1:0.7 :0.16
	90				1:0.85:0.24
					(Experimental.)
Experiment 5 (Fig. 10)	Initial K.....	0.76	0.70	0.33	1:0.92:0.43
	Total K.....	0.580	0.477	0.196	1:0.82:0.34
Experiment 6 (Fig. 11)	Initial K.....	0.44	0.27	0.06	1:0.61:0.14
	Total K.....	0.57	0.348	0.134	1:0.61:0.24

In Figs. 10 and 11 are plotted the experimental points in Experiments 5 and 6 respectively, and the results are summarized in Table VI. For comparison, the initial and the total K's have been calculated. The latter are calculated by the method of least squares and the former directly from the experimental figures. The agreement between these experimental ratios and the expected ratios seems remarkably good when the many complicating factors involved in this type of experimentation are taken into consideration.

[7] The writer wishes to express his gratitude to Professor R. G. Wilson, Massachusetts Institute of Technology, for information on this point and also for valuable criticism of this work.

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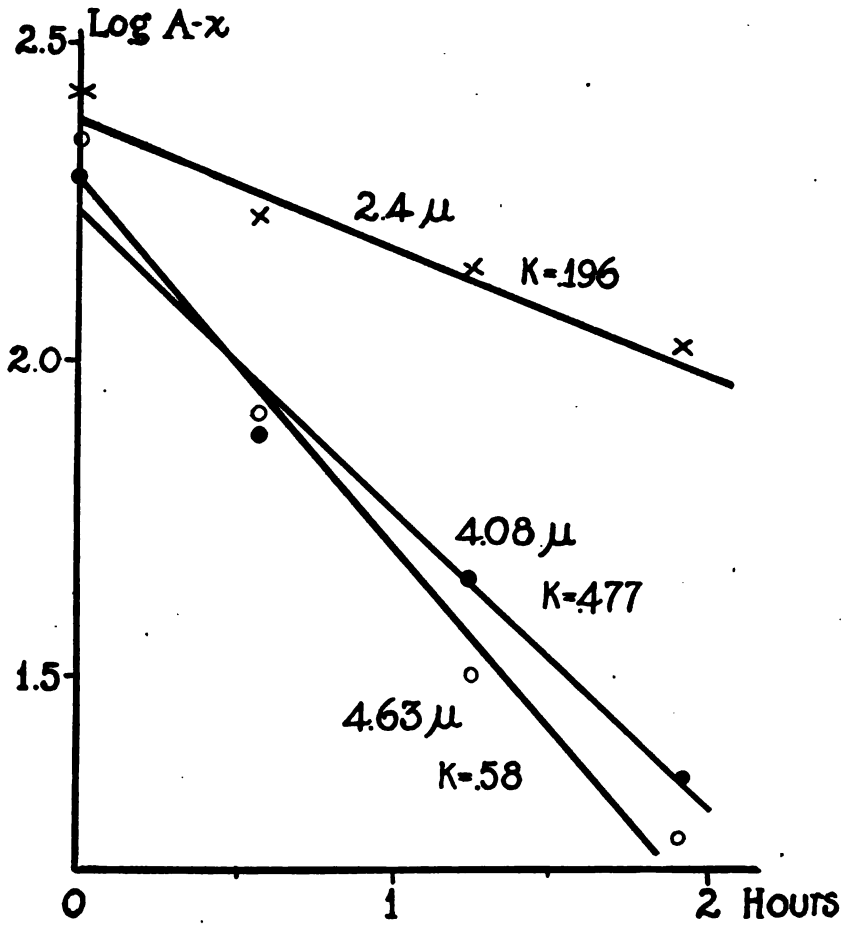

FIG. 10. Experiment 5. Logarithms of number of particles not ingested plotted as ordinates against time as abscissæ. See Table VI for comparison of theoretical and experimental ratios and values of initial K's. Total K's, as plotted, were determined by method of least squares.

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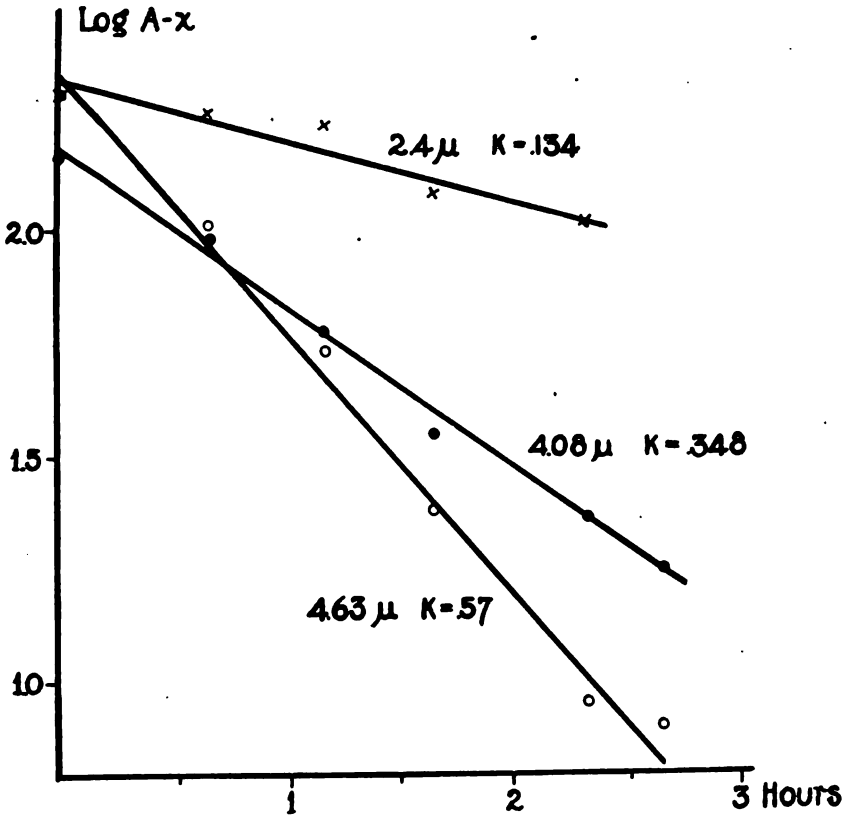

FIG. 11. Experiment 6. Repetition of Experiment 5, Fig. 10. See Table VI for comparisons of theoretical and experimental ratios and values of initial K's.

SUMMARY.

1. A new quantitative method of measuring phagocytosis of solid particles is described.

2. A method of calculating the chances of collision between leucocytes and quartz particles of different sizes is developed.

3. The speed with which three suspensions of different sized quartz particles should be ingested by leucocytes is predicted from the calculated chances of collision, and the prediction is verified experimentally.

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4. The formula for the chances of collision is also verified by varying the speed of rotation of the tubes in which the phagocytic mixtures are incubated.

The advice and assistance of Doctor C. K. Drinker and Doctor C. K. Reiman of this department are gratefully acknowledged.

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THE PHAGOCYTOSIS OF SOLID PARTICLES.

II. CARBON.

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(Received for publication, January 15, 1921.)

In a former paper (1) some determinations were reported of the rates of ingestion of three different sizes of quartz particles by leucocytes of the rat. It was shown that the rate of phagocytosis depends upon the chances of collision between a leucocyte and a particle of quartz. In this paper similar experiments on the phagocytosis of carbon particles of two sizes are reported.

Carbon Suspensions.

The method of preparing the carbon suspensions was essentially the same as that already described for quartz. The carbon used was one of the charcoals prepared from coal during the war by the Chemical Warfare Service and activated by treatment with superheated steam. It was washed repeatedly in distilled water during the process of removing, by means of the centrifuge, the particles too small to use, and was washed once each time before using. It may, therefore, be considered free from easily soluble material.

The carbon suspensions were considerably more sensitive to agglutination than the quartz. It was, for example, impossible to resuspend a suspension of carbon, after it had settled out either by gravity or centrifugal force, without getting some agglutination. The suspensions were agglutinated at once by the addition of tap water and they were somewhat more stable in slightly alkaline than in slightly acid solutions. Non-sterile suspensions agglutinate more readily than sterile ones. Acacia must be added to a suspension to keep it uniform and stable for more than a few hours. Fortunately

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the hemoglobin and serum proteins act fairly efficiently as protective colloids, and the normal reaction of blood, at which these experiments were done, is slightly alkaline. Otherwise, it would be impossible to keep the particles distinct in 0.9 per cent sodium chloride with the leucocytes. Even so, the agglutination of the carbon was a very serious complicating factor in these experiments, more so than with quartz.

In view of the readiness with which these uniform suspensions agglutinate, it is a significant fact that in the stock suspensions, from which the large particles have not yet been removed, all the particles are never agglutinated even after being kept for nearly a year without acacia. It would seem as if coagulation must reach an equilibrium and stop. It is certain that there is always an abundance of the smallest sized particles which are not clumped and can readily be separated from the larger particles and the clumps by settling.

Chances of Collision with Leucocytes.

The diameters of the particles in the two suspensions and the diameters of the leucocytes were measured microscopically. The variation in these diameters is shown in three frequency curves in Fig. 1. On account of the lack of uniformity of these suspensions the chances of collision between leucocytes and particles could not be calculated from the average diameter and velocity of all the particles in the suspension. It was, therefore, necessary to divide each frequency curve, as given in Fig. 1, into sections and calculate the chances of collision, R, for each possible combination, by the formula $R = (C + P)^2 (Vp - Vc)$ (1). Here the first term is the square of the sum of the diameters of the cell and particle, and allows merely for the larger target offered by a large particle. The second term is the difference in velocity between the cells and particles. Since the velocities of the cells and particles in this case were nearly equal, a small error in Vp or Vc would make a large error in R. It was, therefore, necessary to divide the carbon suspensions into six groups each to obtain sufficient accuracy in the calculation. The leucocytes were divided into three groups as described in the previous paper. The total chances of collision were then given by the sum of the 18 (6×3)

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group chances of collision, each multiplied by the percentage of cells and of particles in that particular group. The data from which the chances of collision were taken are given in Table I together with the chances of collision of each suspension.

It will be noted that in calculating the chances of collision the velocity was determined experimentally and was not calculated from the diameter and density. This was done because of the fact that the velocity of carbon particles is not proportional to the density of dry carbon which is full of capillary spaces, but to its density with

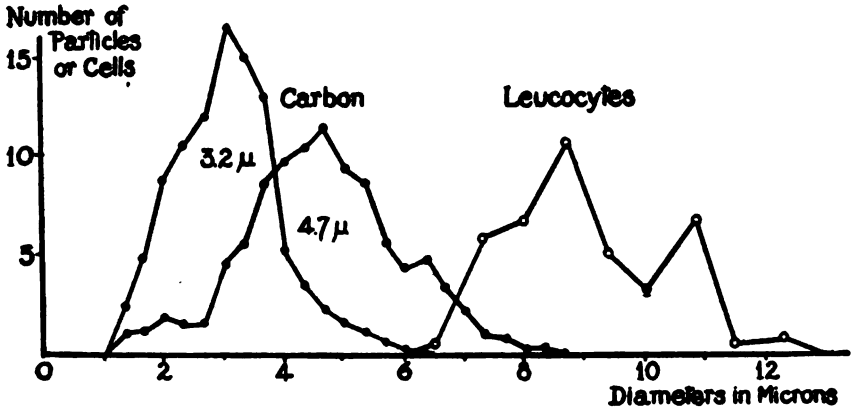

FIG. 1. Frequency curves showing the variation in the diameters of two carbon suspensions and the leucocytes. Curves are plotted on such a scale that the subtended areas are approximately equal. Ordinates represent number of particles for a given interval of the abscissæ, which represent the diameters in microns. Points plotted are experimental points. In the case of the carbon suspensions they are smoothed by averaging each point with the two adjacent points.

these spaces full of water; and the latter is not easily determined. However, having obtained the velocity of each group directly, without using any value for the density, it is possible to reverse the process and calculate both the density and the volume of the capillary spaces, as a check on the accuracy of the measurements.

By a simplification of Stokes's law for the velocity, V, of a particle of diameter, P, and density, D, in a medium of density 1.00 and viscosity 0.010 under the acceleration of gravity, we obtain the equation

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$$D = 1 + \frac{5.1\ V}{P^2}$$

Substituting in this equation values for the average velocities and average diameters of the two suspensions (0.87 ± 0.12 and 2.19 ± 0.18 cm. per hour for the 3.2μ and 4.7μ respectively), and solving for D, we obtain 1.43 ± 0.06 and 1.50 ± 0.04. This is the density with capillary spaces full of water.

TABLE I.*

Chances of Collision between Leucocytes and Carbon Suspensions.

3.2 μ				4.7 μ			
Particles in group.	Group diameter.	Group velocity.	Group R.	Particles in group.	Group diameter.	Group velocity.	Group R.
per cent	μ	*cm. per hr.*		*per cent*	μ	*cm. per hr.*	
14.3	1.8	0.27	24.9	5.3	1.9	0.35	9.1
36.2	2.7	0.59	58.6	14.0	3.2	0.99	16.2
39.4	3.5	1.01	46.6	29.6	4.08	1.61	17.9
7.3	4.7	1.78	5.1	27.9	5.0	2.39	35.6
2.04	5.6	2.58	3.7	13.3	5.94	3.36	48.6
1.0	6.8	3.8	5.2	10.0	6.8	6.5	120.8
Total chances of collision = 144.1				Total chances of collision = 248.2			

* Chances of collision, R, with cells of the two carbon suspensions. The group diameters were obtained by averaging the squares of the individual diameters as measured in the microscope. Group velocities were calculated in proportion to the squares of the group diameters and so that their weighted average velocity should be equal to the average velocity of the suspension as determined experimentally by the stop-cock method; *i.e.*, to 0.87 ± 0.12 and 2.19 ± 0.18 cm. per hour for the 3.2μ and 4.7μ suspensions, respectively. The R group or chance of collision is given by the formula

$$R \text{ group} = (R_{1a}\ P_a + R_{1b}\ P_b + R_{1c}\ P_c)\ \frac{P_1}{100}$$

where P equals the per cent of particles or cells in designated group, subscripts a, b, and c refer to the three groups of cells, and the numerical subscripts refer to the groups of particles.

From these figures may be calculated the cubic centimeters of capillary space, x, in the carbon per gram of dry weight, as follows:

$$D = \frac{1.81 + 1.81x}{1 + 1.81x}$$

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1.81 being the density of dry carbon. Whence, introducing the calculated values for D and solving, we get $x = 0.49 \pm 0.09$ and 0.34 ± 0.07, the average being 0.42 cc. of capillary space per gram of carbon. Though the variation is relatively large it is indicative of the general accuracy of the calculations that this is the exact figure given for the volume of the capillary spaces in charcoal by Cude and Hulett (2), from a study of a large series of charcoals used by the Chemical Warfare Service.

It is possible, then, to predict the behavior of the leucocytes toward the particles of these suspensions when they are incubated together. The predictions are: (1) that the large particles will be taken up more rapidly than the small particles, the initial ratio of the rates of ingestion being as 248 : 144 = 1 : 0.58; and (2) that this ratio will probably increase with time. The latter prediction is illustrated by the following figures for the ratio of the chances of collison of the two suspensions at such times during the reaction that 25, 50, and 75 per cent of the larger particles are ingested.

Change in Ratio Predicted during Reaction.

4.7 μ particles ingested.	Ratio. $R_{4.7\,\mu} : R_{3.2\,\mu}$
per cent	
0	1:0.58
25	1:1.07
50	1:1.26
75	1:1.42

This change in ratio is largely due to lack of uniformity of the 4.7μ suspension, the largest particles having an abnormally high chance of collision as shown in Table I. The prediction is, however, merely a probability since the assumption upon which it is based, namely that the leucocytes remain unchanged in concentration, can never be completely true. However, it may be noted that the experiments largely verify these predictions.

Method of Measuring Phagocytosis.

The detailed technique of these experiments is given in a previous paper (1). It is sufficient to state here that mixtures consisting of washed leucocytes from rats, carbon suspension, and serum were

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incubated at 37°C. in small test-tubes (8 mm. inside diameter), placed horizontally on a drum revolving uniformly about its horizontal axis. The speed of rotation was usually about 15 revolutions per minute. In these experiments no precautions were taken to prevent an air bubble from traveling up and down in the tube as it revolved. Samples were removed at intervals and counts were made of the *number of particles not ingested* by the leucocytes.

The measure of phagocytosis desired is, then, the per cent of the collisions occurring between cells and leucocytes which result in ingestion. But the number of leucocytes being the same in any one experiment, the *relative* number of collisions is proportional only to the number of particles. If the percentage of collisions resulting in ingestion is constant and the concentration of cells does not change, the rate of ingestion will always be proportional to the number of particles still free to collide, and the velocity constant, K, will be given by the equation for a monomolecular reaction, $K = \frac{1}{t} \log \frac{A}{A - x}$ where A is the original number of particles and x is the number ingested in time, t. Here the relative value of K is dependent only on the percentage of collisions resulting in ingestion, and is, therefore, the measure of phagocytosis desired. K has been determined in practice by plotting the logarithms of the number of particles still free, *i.e.* $\log A - x$, against time and determining the slope graphically. The points should lie on a straight line if K is constant. This is more accurate than calculating K directly from the formula, since the formula attaches special importance to A which is known no more accurately than the rest of the points.

Where K is not constant the initial value of K only is determined. A *decrease* in K with time may be due to:

1. Decrease in the number of cells by agglutination.[1]
2. Decrease in the capacity of cells for particles.
3. Lack of uniformity in the suspensions resulting in decrease in chances of collision as larger particles in each suspension are ingested more rapidly than the small particles.

[1] It can be shown that in calculating the chances of collision the decrease in the number of cells by agglutination is not compensated for by the increase in velocity and size of the resulting clumps.

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4. Decrease in activity of cells due to internal changes or changes in environment.

An *increase* with time may be due to:

5. Increase in velocity of cells caused by increased density after ingestion of particles.

6. Increase in activity of cells by internal changes or changes in environment.

Of these factors, No. 3 can be predicted by measurements of the particles in the suspension and has no effect if thoroughly uniform suspensions are used. Nos. 4 and 6 are probably negligible. Nos. 1, 2, and 5, then, are the significant factors. No. 1 is presumably the same for each carbon suspension in any one experiment so that it does not vitiate the comparison. Nos. 2 and 5 are small if the number of cells is large. In the majority of experiments there were from one to two particles per cell in the most concentrated suspension, probably never over three, and frequently less than one. Since each cell could take up perhaps five large particles and fifteen small ones they could hardly be appreciably filled up. It seems valid to use the total K, where K is constant, as well as the initial K for comparison. Usually the total K does not differ from the initial K by more than the experimental error. The latter is, of course, the more inaccurate as it depends upon only two points, while the total K is the average of all points. It is, perhaps, surprising that K should be so constant. This must indicate that the disturbing factors are in such cases comparatively small and that those causing an increase in K partly compensate for those causing a decrease.

In Table II the results of five such comparisons of rates of ingestion of the two suspensions of carbon are given. The results for three of these experiments are plotted in Figs. 2, 3, and 4. The other two experiments are somewhat less reliable and are included merely for completeness.

It must be admitted that the agreement between the theoretical initial ratio and the average experimental ratio is better than the variation in the individual ratios warrants. It will be seen that in Figs. 2 and 3 the second prediction is fulfilled as well as the first, for the slope of the curve representing the ingestion of the 4.7μ particles continually decreases, while that of the 3.2μ particles remains con-

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TABLE II.

Comparison of Experimental and Theoretical Rates of Phagocytosis.

Experiment No.	Initial K.		$\frac{K_{3.2\mu}}{K_{4.7\mu}}$	
	4.7 μ	3.2 μ		
1	0.36	0.12	0.30	Fig. 2
2	0.60	0.38	0.63	" 3
3	0.21	0.16	0.76	" 4
4	0.125	0.10	0.80	Not figured.
5	0.88	0.32	0.36	" "
Average			0.57	
Theoretical			0.58	

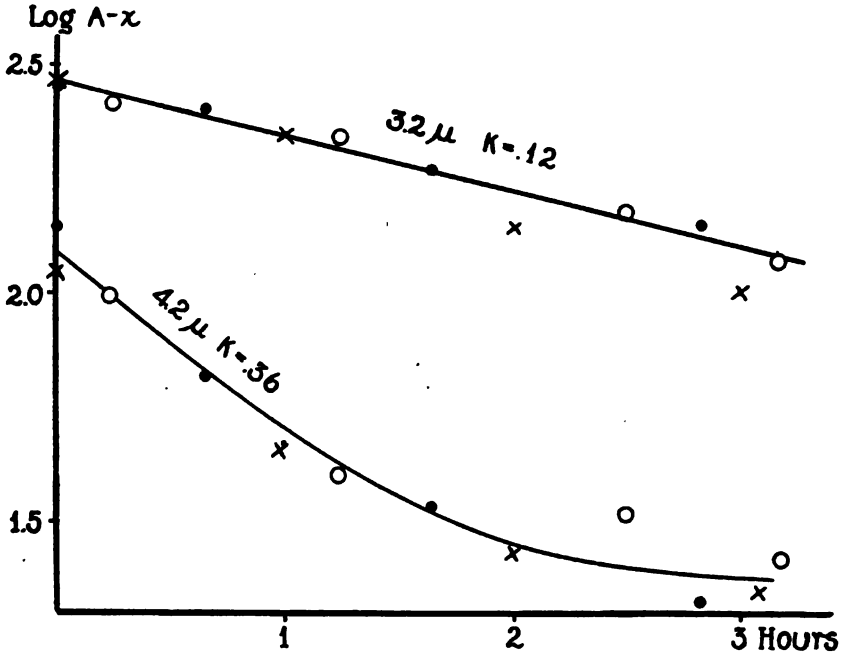

FIG. 2. Ordinates represent logarithms of the number of particles not yet ingested. Abscissæ represent time in hours since the beginning of the experiment. Points from three separate but simultaneous experiments are plotted for each curve. Experimental ratio $\frac{K_{3.2\mu}}{K_{4.7\mu}}$ is 0.3; theoretical ratio is 0.58. The fact that $K_{4.7\mu}$ is not constant causes this ratio to increase as predicted.

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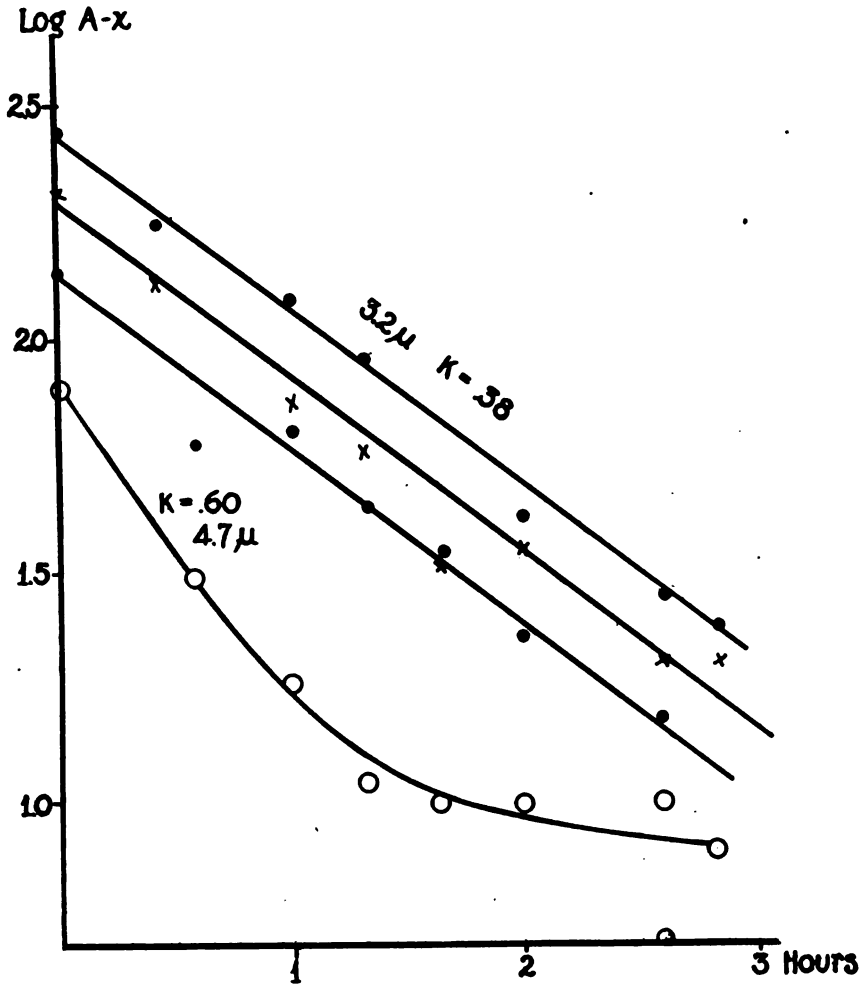

FIG. 3. Curves showing the rates of ingestion of 3.2μ and 4.7μ carbon particles. The logarithms of the number of particles not ingested are plotted (ordinates) against time (abscissæ). All three concentrations (4, 3, and 2) of the 3.2μ particles give $K = 0.38$ approximately. Experimental initial ratio $\frac{K_{3.2\mu}}{K_{4.7\mu}} = 0.63$; theoretical ratio is 0.58. This ratio increases with time due to the decrease in $K_{4.7\mu}$ as predicted.

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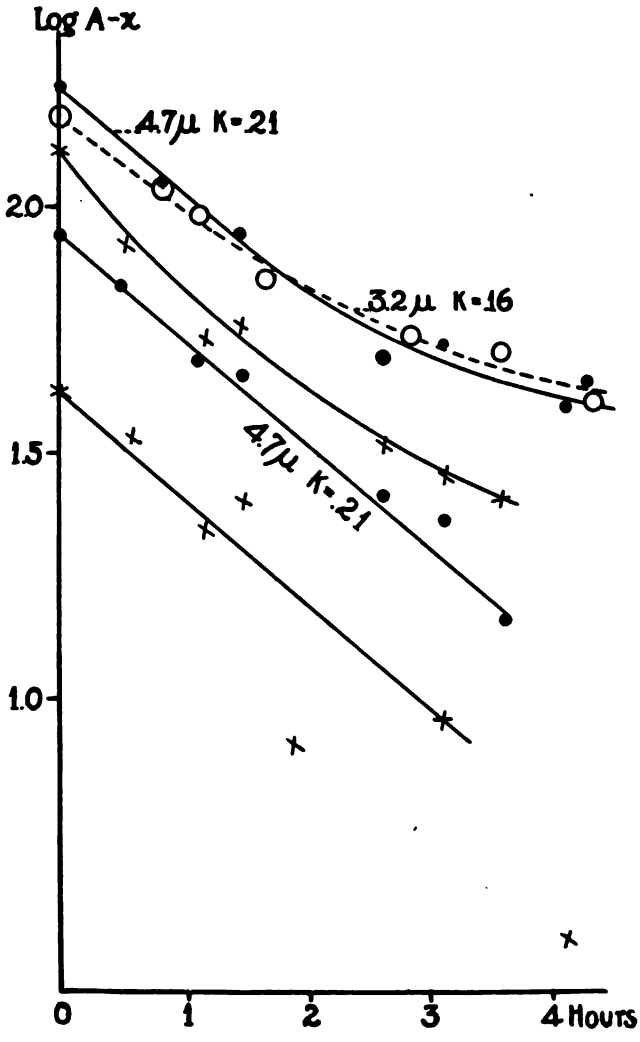

FIG. 4. Curves showing logarithms of numbers of 3.2μ and 4.7μ particles not ingested (ordinates) plotted against time (abscissæ). Four concentrations (4, 3, 2, and 1) of the 4.7μ particles were used. The best initial K is 0.21 which is well satisfied except in one case. Experimental ratio $\frac{K_{3.2\mu}}{K_{4.7\mu}} = 0.76$; theoretical ratio is 0.58. This ratio does not increase during the experiment.

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stant throughout the experiment. Fig. 3 is an exception, however, since K decreases for the 3.2μ particles and the higher concentrations of the 4.7μ particles in about the same proportion. There is, therefore, no increase in the value of $\frac{K_{3.2\mu}}{K_{4.7\mu}}$. In the two experiments which were not calculated, Experiment 4 showed an increase in this ratio while Experiment 5 showed none.

There are two causes for the variations in the experimental result: (1) The suspensions used were not identical, for the material for each experiment was separately prepared from the stock suspensions by removing the large particles by centrifugalization. (2) There was a slight tendency to aggregation on the part of the carbon which was more marked in some cases than in others.

In Fig. 3, three different concentrations of the 3.2μ particles were used, in the proportions 4 : 3 : 2, all of which have practically the same K throughout. In Fig. 4, similarly, four different concentrations of the 4.7μ particles were compared, all of which give approximately the same K. This is a better proof that the reaction follows the law for a monomolecular reaction than the constancy of K in a single experiment because of gradual aggregation of the cells.

While the agreement between theoretical and experimental ratios in these experiments is admittedly rough, it seems surprisingly good considering the difficulties involved. It is at least accurate enough to discourage any attempts to measure the greater difficulty of ingesting a large particle compared to a small one by this method, which was the original object of these experiments, because the limiting factor is the availability of the particles, not the phagocytic capabilities of the leucocytes.

It should perhaps be emphasized here that a certain proportion of the particles counted as phagocytized are merely stuck on the outside of clumps of cells where it is impossible to distinguish them from those inside. This does not occur with dead cells, however, and is rightly regarded as the first stage in phagocytosis.

Phagocytosis of Bacteria.

It is interesting to inquire at this point whether the phagocytosis of bacteria also follows the law for a monomolecular reaction, as does

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the phagocytosis of solid particles. This can only be determined if the number of bacteria ingested in proportion to the number of bacteria present is known. In most experiments on phagocytosis of bacteria no counts have been made of the total number of bacteria present. The only data known to the writer from which this point can be determined are those of Madsen and Watabiki (3) and of Ledingham (4). The former measured the number of bacteria ingested as a function of time. Their curves follow the law of a monomolecular reaction fairly well if all the bacteria not ingested at the close of the experiment, *i.e.* when phagocytosis has ceased, are disregarded. The rate of phagocytosis is, therefore, not proportional to the concentration of the bacteria present.

Ledingham did for phagocytosis of bacteria what was done in Figs. 3 and 4 for the phagocytosis of carbon. He measured the number of bacteria ingested in unit time by a given number of cells from a series of bacterial suspensions of varying known concentrations. If the reaction had been of the monomolecular type the percentages ingested at each concentration should have been equal, just as all the K's in Figs. 3 and 4 were equal for the same size particle: He found, however, that as the concentration of bacteria decreased, the percentage ingested first increased and then decreased. There is, therefore, no evidence that the phagocytosis of bacteria follows the law for a monomolecular reaction. If, as Ledingham believes, the comparatively slight decrease in the percentage ingested in low concentrations of bacteria is due to experimental error, the decrease in higher concentrations may be due to increasing concentrations of some substance given off by the bacteria which is toxic to the cells.[2]

[2] In these experiments Ledingham's object was to prove that phagocytosis of bacteria obeys Freundlich's exponential formula for adsorption, but it is hard to see how the formula could have any significance for phagocytosis even if applicable. He also tries to show that the adsorption of opsonin from serum by bacteria follows the same law, but his measure of the concentration of opsonin adsorbed is mathematically incorrect. He assumes that the opsonin adsorbed by the bacteria is proportional to the difference in the number of bacteria ingested by a given number of leucocytes in the presence of an opsonin solution, before and after that solution has been treated with a given bacterial emulsion (which adsorbs some of the opsonin) and freed from it by centrifugalization. His own figures show, however, as he himself observes, that the number of bacteria ingested is

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Ledingham himself describes "lytic changes" in these leucocytes which he ascribes to the "leucotoxic action of unneutralised bacterial extract." This supposition might also explain the fact observed by Madsen and Watabiki that the rate of phagocytosis decreases more rapidly than the concentration of bacteria, the cumulative effect of the toxic substance finally preventing phagocytosis altogether before all the bacteria are ingested.

Other Experiments on Phagocytosis of Carbon.

The Effect of Serum.

Hamburger (5) found that the carbon particles which he used with horse leucocytes were ingested just as well in pure sodium chloride as in sodium chloride and serum. Fig. 5 shows that this was distinctly not the case in these experiments. In this figure Hamburger's measure of phagocytosis was used; *i.e.* the percentage of cells containing carbon. These values are plotted as ordinates in Fig. 5 against time as abscissæ. Two controls, one without serum and one with serum, heated to 56°C. for 40 minutes, showed almost no phagocytosis. This is the usual result obtained by previous workers on phagocytosis of solid particles, although there is considerable disagreement. Hamburger finds serum necessary for the phagocytosis of starch but not of carbon.

Porges (6) found that starch is taken up from isotonic solutions without serum as well as with serum, but the accelerating effect of serum became evident in hypertonic solutions.

Ouweleen (7) concludes that two substances are necessary for the phagocytosis of starch: (1) a thermolabile constituent of serum which is absorbed on the starch and causes adhesion of the starch to the leucocyte; and (2) a substance causing the adhering particle to be

not a linear function of the opsonin concentration. From his figures for the number of bacteria ingested in the presence of known concentrations of opsonin, it is possible to calculate graphically what the true concentration of opsonin must be after adsorption to account for the observed number of bacteria ingested. Using figures so obtained the adsorption formula no longer applies. In only one experiment, however, were his data sufficiently complete to make this method of analysis possible.

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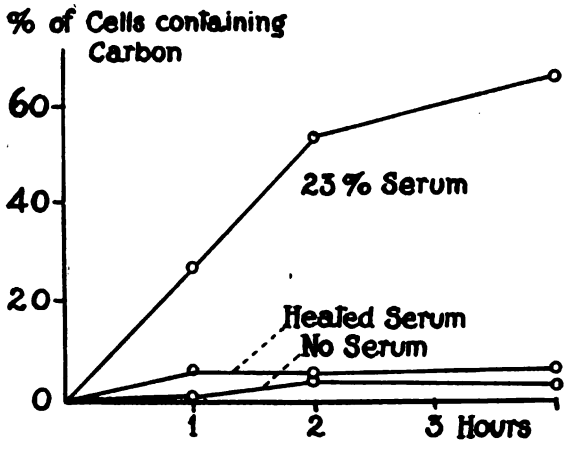

FIG. 5. Curves showing that serum increases phagocytosis of carbon and that its effectiveness in this respect is destroyed by heating to 56°C. for 40 minutes.

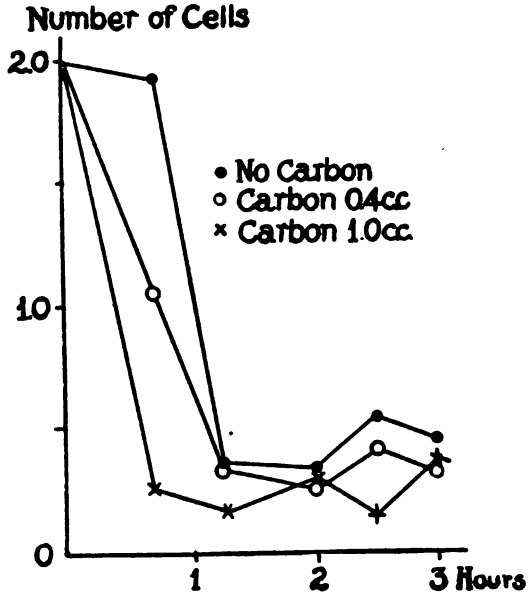

FIG. 6. The number of unclumped cells ($\times 10^4$ per mm.^{3}) plotted as ordinates against time in hours as abscissæ. Curves show that the cells clump more rapidly in presence of 3.1 and 1.25 $\times 10^4$ particles of carbon per mm.^{3} The same effect was observed in two other unpublished experiments in which counts of the cells were made.

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ingested for which proteins (as ovalbumin) other than serum proteins may be used.

When cells are incubated in dilute serum with carbon they agglutinate more rapidly than when carbon is omitted. A typical experiment is plotted in Fig. 6. These data were taken from an early experiment when the cells from the peritoneal exudate were not washed in sodium chloride. Under these conditions agglutination of the cells occurred to a considerable extent, since it was impossible to add enough sodium citrate to prevent it without preventing phagocytosis as well. The degree of agglutination in this figure is, therefore, not characteristic of the other experiments in this paper.

Three curves are plotted showing the number of cells still unclumped without carbon, and with the addition of two concentrations of carbon. More cells are always free in the control. The obvious conclusion is that the process of ingesting carbon makes the cells more sticky so that they agglutinate more readily.

The result of the more rapid agglutination of cells in the presence of carbon is that the percentage of cells containing carbon is higher for the clumped cells than for the unclumped cells. This constitutes, therefore, a serious objection to the use of the percentage of cells containing carbon as a measure of phagocytosis (8). The reaction may appear to have come to a standstill when the aggregation of carbon-containing cells is merely keeping pace with the ingestion. Kite and Wherry (9) have also observed that clumped cells contain more bacteria than unclumped cells. This might be due, however, to the greater velocity of clumps of cells, the velocity increasing roughly as the two-thirds power of the number of cells in the clump. Neufeld and Rimpau (10), however, reported in 1905, from well controlled experiments, that phagocytizing leucocytes clump more rapidly than control leucocytes where phagocytosis is prevented by omission from the mixture of either the immune serum or the bacteria. This seems, therefore, to be a general phenomenon.

In experiments with unwashed cells the behavior of the cells is interesting though the results are worthless as accurate measures of phagocytosis. A typical experiment is plotted in Figs. 7 and 8. In Fig. 7 the number of cells still unclumped is plotted as ordinates against time as abscissæ. The percentage of these cells which con-

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tained carbon is plotted as ordinates in Fig. 8. In 26 per cent serum nearly all the cells had clumped up in 15 minutes, after which they began to creep out of the clumps until at the end of 4 hours nearly one-half the original number was again free. In pure 0.9 per cent sodium chloride (control) there is least clumping and least phagocytosis. Similarly, in 26 per cent serum where clumping is most

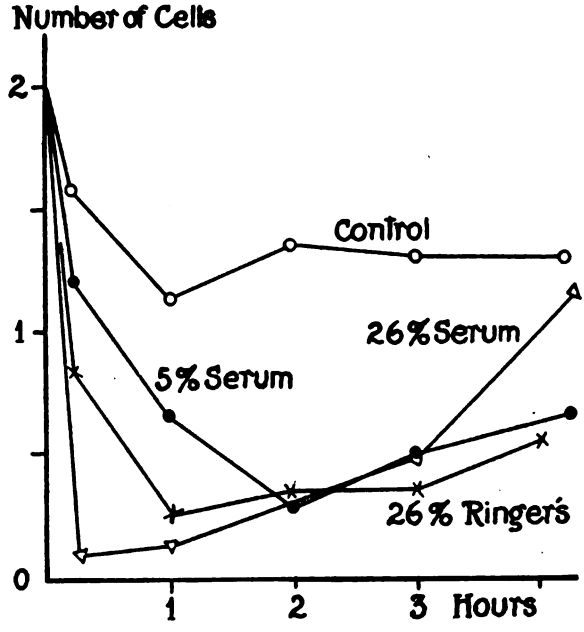

FIG. 7. Ordinates and abscissæ as in Fig. 6. Curves illustrate the effect of serum and an equivalent concentration of Ringer's solution on the clumping of leucocytes. After the first rapid agglutination, the cells begin to creep out of the clumps again.

rapid, phagocytosis is also most rapid. Part of the effect of serum is due to its calcium, since the same amount of Ringer's solution also increases both phagocytosis and clumping, though to a lesser degree. The small percentage of free cells containing carbon at the end of the 1st hour (Fig. 8) is probably due to the more rapid clumping of such cells, as already described (Fig. 6).

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In general it was found in these experiments that anything which increased clumping also increased phagocytosis and *vice versa*. Thus, the higher the concentration of sodium citrate, the less the phagocytosis and the less the clumping.[3] With washed cells from which all the fibrinogen has been removed, the number of free cells shows only a gradual decrease during the experiment.

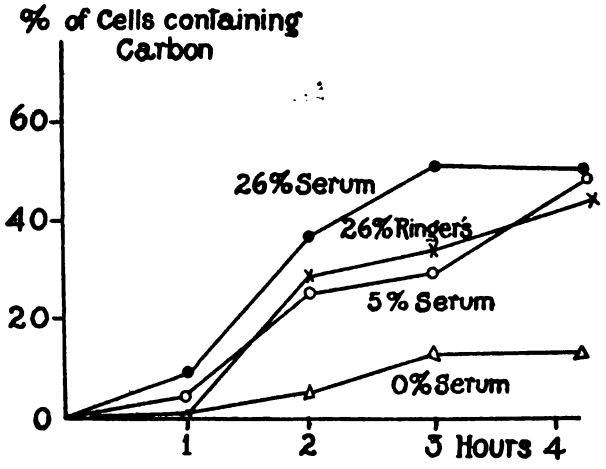

FIG. 8. Ordinates represent the per cent of the unclumped cells counted in Fig. 7, which contained carbon. Curves show that serum which accelerates clumping cells also accelerates phagocytosis. The similar effect of Ringer's solution shows that the calcium in serum is partly responsible for its effect.

SUMMARY.

1. By measurements of the diameter and velocity of leucocytes and of the particles in two carbon suspensions, the relative rates of ingestion of the two suspensions by the leucocytes are predicted and the predictions verified experimentally.

2. The results indicate that 4.7μ particles of carbon are ingested as readily as 3.2μ particles. The more rapid *apparent* rate of ingestion of the 4.7μ particles is due to their greater *availability* rather than the greater *capability* of the leucocytes.

[3] McJunkin (McJunkin, F. A., *Arch. Int. Med.*, 1918, xxi, 59) has used the ability of white blood corpuscles to ingest carbon in varying concentrations of sodium citrate as a means of classification.

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3. There is almost no phagocytosis of carbon in absence of serum or in heated serum.

4. The clumping of unwashed leucocytes is accllerated by serum and by the ingestion of carbon.

5. The available evidence indicates that the phagocytosis of bacteria does not follow the law for a monomolecular reaction, possibly because of the toxic effect upon the leucocytes of bacterial extracts.

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AN INVESTIGATION INTO THE CAUSE OF THE SPONTANEOUS AGGREGATION OF FLAGELLATES AND INTO THE REACTIONS OF FLAGELLATES TO DISSOLVED OXYGEN.

PART I.

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The Phenomena of Spontaneous Aggregation and Band Formation.

It is well known that many flagellate and ciliate Protista form spontaneous aggregations. That is, when a drop of water containing the organisms is mounted on a slide for examination under the microscope the flagellates or ciliates are frequently seen to collect into clumps or masses. The center of such a collection may be a piece of some solid present in the water, or there may be no such visible focus. It is the second of the two cases that is here termed spontaneous aggregation, and the present investigation was undertaken with the object of finding out the cause of this phenomenon. The discovery of the cause then led on to the more general question of the relation of the organisms to varying amounts of dissolved oxygen.

Throughout the investigation the same species of flagellate was used; namely, *Bodo sulcatus*. It was originally described by Mereschkowsky.[1] For the present investigation the flagellate was obtained by taking grass from the garden behind the laboratory at Plymouth and steeping it in tap water. The best material was obtained from 6 day old cultures. The flagellates were then most abundant without being too much mixed with other organisms. In cultures older than 6 days there were too many bacteria present and, still later on, too many ciliates.

[1] Mereschkowsky, C., *Arch. mikr. Anat.*, 1879, xvi, 153.

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The phenomenon of spontaneous aggregation is best studied as follows: A square cover-glass supported by wax feet at its four corners, or, better still, by short pieces of glass rod previously cemented to the corners, is placed on a dry slide. Some liquid from a *Bodo* culture is then run in from a pipette beneath the cover-glass until it just fills the space between the latter and the slide. At first the flagellates are evenly scattered throughout the preparation (Fig. 1) but they do not remain so for an indefinite time. At the end of an

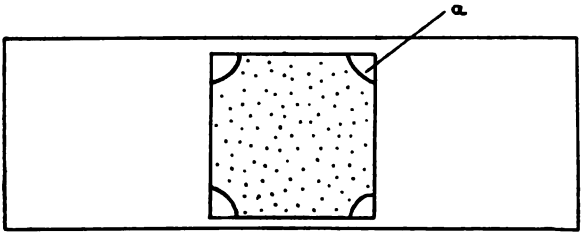

FIG. 1. Slide and supported cover-glass at beginning of an experiment, showing flagellates evenly scattered through the liquid beneath the cover-glass. In all diagrams the density of the dots represents the density of distribution of the flagellates. *a*, wax support to cover-glass. Figs. 1 to 5 are successive stages of one experiment.

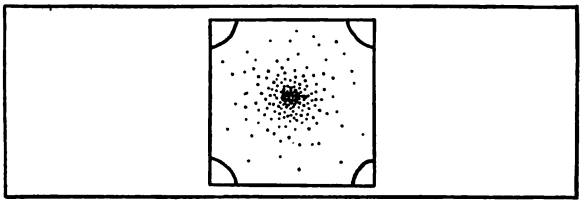

FIG. 2. Formation of central aggregation of flagellates.

interval which may be 2 minutes or 2 hours they begin to collect into one or more groups towards the center of the cover-glass, the size of these aggregations increasing until they contain most of the *Bodo* present in the liquid (Fig. 2). The flagellates in the aggregations are in intense movement. There is no solid body forming the center of a collection and indeed the presence of any such object is purposely avoided by filtering the suspension of *Bodo* through fine bolting-silk before making the preparation. Under a square cover-glass measuring $\frac{7}{8}$ inch $\times \frac{7}{8}$ inch, which was the size used in the

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experiments, one central aggregation is usually formed but sometimes there are several, always near the center.

After an aggregation has been in existence for a short time a clear space free from flagellates appears at its center. The central space enlarges until the flagellates come to lie in a circular band around it, this band being easily visible to the naked eye (Fig. 3). The increase in size of the clear area goes on steadily, and as the band of flagellates surrounding it approaches the edges of the cover-slip the sides of the band become flattened (Fig. 4) and then, still enlarging, the band gradually comes to form a square, the sides of which are parallel to

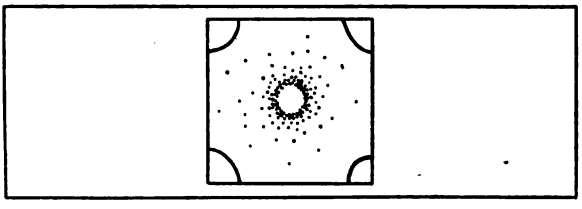

FIG. 3. Appearance of region free from flagellates in the center of the aggregation.

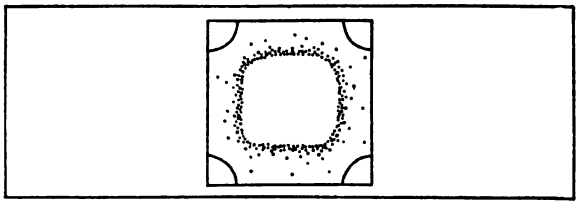

FIG. 4. Circular band of flagellates becoming square.

the sides of the cover-glass. When this square-shaped band of flagellates has reached a certain distance from the edges of the preparation, it becomes stationary, the central clear area no longer increasing in size (Fig. 5). The distance of the final position of the band from the edge of the cover-slip depends on the height of the latter above the slide; the band comes to lie the nearer to the edge the lower the cover-slip. The great majority of the flagellates present in the preparation are in the band, but nevertheless there are always a few swimming in the region between the band and the edge of the cover-glass.

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A grain of sand lying in the path of the band of *Bodo* as it advances from the center towards the edges of the preparation has no effect on the band: the latter approaches and passes the grain of sand without being deflected. An air bubble, however, keeps the band at a distance from it. If the bubble lies sufficiently far in from the edge of the cover-glass the advancing band of flagellates becomes bent inwards to form a bay enclosing the bubble (Fig. 6). As the main band continues to move outwards the horns of the bay meet so that an inner ring of flagellates is left behind surrounding the bubble

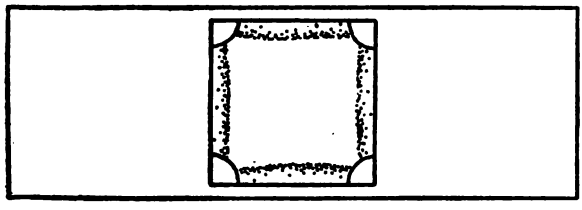

FIG. 5. Equilibrium position of flagellate band.

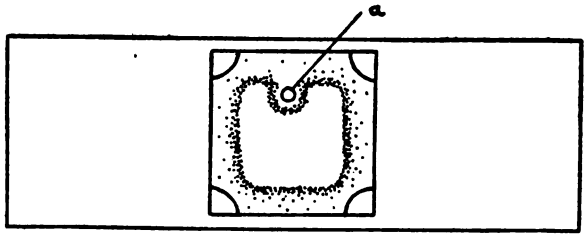

FIG. 6. Air bubble keeping back an advancing flagellate band. *a*, air bubble. Figs. 6, 7, and 8 are successive stages of one experiment.

(Fig. 7). The bubble, then, behaves to the band just as the water-air surface at the edges of the preparation does: it keeps the flagellate band at a certain distance from it. But there is this difference between the two cases, that whereas the main band comes to a halt and remains stationary at a certain distance inside the edges of the preparation, the band encircling the bubble slowly approaches the latter. This continues until the flagellates come into contact with the surface of the bubble itself (Fig. 8), where they remain for a short time and then dissipate, swimming out to join the main band.

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If a preparation such as has been described, in which the square-shaped band of *Bodo* is established with its sides parallel to the edges of the cover-glass, is kept in a moist chamber and examined again on the following day, it will be found that the band has retreated somewhat from the edges of the cover-slip. On the following day again the band will be further in still and will have taken on a circular instead of a square form. Later on all the flagellates will be clumped in one mass at the center of the slide, after which they will gradually dissipate to become evenly scattered throughout the liquid again. In fact the *Bodo* band goes through the same series of changes which

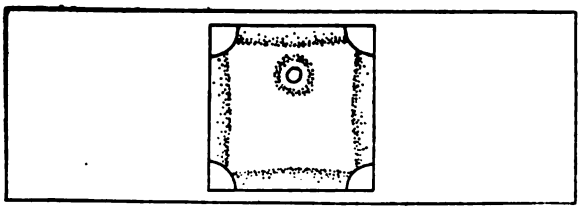

FIG. 7. Main flagellate band reaches its equilibrium position having left behind an inner ring surrounding air bubble.

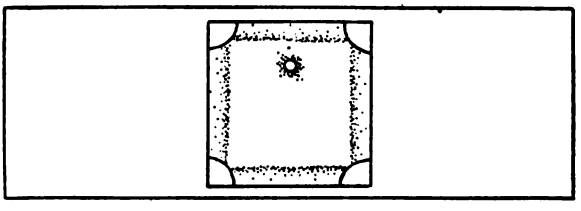

FIG. 8. Flagellates around air bubble advance to its surface.

it originally underwent in its formation but in the reverse order. Through all the series of changes the organisms continue in full motile activity. The retiral of the band from the outside may be hastened by placing the slide in an ice chest. In the course of a few hours only, the square band will have become a circular band situated near the center of the preparation. The swimming activity of the *Bodo* is, incidentally, not noticeably decreased by the low temperature. If, on the other hand, the moist chamber containing the preparation is kept at a higher temperature than that of the laboratory, the band retires from the edges much more slowly, if at

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all. Furthermore, a flagellate band which has been caused to retire inwards by keeping the preparation at a low temperature will move outwards again as soon as the slide is replaced in the higher temperature of the laboratory.

The Cause of Aggregation and Band Formation.

In seeking for the causes of these phenomena, there are three questions to be asked.

1. What is the cause of the aggregation of the flagellates at the center of the preparation?

2. Why does an aggregation become a band surrounding a region clear of flagellates, which continuously increases in size?

3. Why does the central clear region cease to increase in size when the band of flagellates bordering it has reached a certain distance inside the air-water surface at the edge of the cover-slip?

The different condition which arises at the center of the preparation, attracting thither the flagellates, must be due to some substance or substances produced by the organisms themselves. It cannot be any excretory product of the nature of a solid in solution or a liquid, for, since the flagellates were originally evenly scattered throughout the preparation, the newly produced substance too would be evenly distributed and would tend neither to attract nor to repel the organisms in any particular region. The changed condition in the center of the preparation must be due to some volatile substance in solution, which at the free edges of the liquid is effecting an exchange with the atmosphere, either going into or out of solution. The only gases in solution which would be changed in amount by the living organisms are oxygen and carbon dioxide; the amount of the former present in solution in the water must continuously decrease and the amount of the latter increase. These changes in the concentrations of dissolved oxygen and carbon dioxide will take place more rapidly in the central region of the liquid than at its edges; for at the edges the oxygen used up by the flagellates will be replaced from the atmosphere, while the carbon dioxide produced by them in this region will go out of solution into the atmosphere as soon as its tension in solution rises above the value corresponding to its partial pressure in the

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atmosphere. In the center of the preparation no exchange with the air can take place, so that here the concentration of dissolved oxygen will decrease and that of carbon dioxide increase most rapidly.

It seems probable, then, that the flagellates collect in the central region because they are attracted either (*a*) into a region of higher hydrogen ion concentration, or (*b*) into one where the concentration of dissolved oxygen is lower. Which of these alternative explanations is the correct one? It was attempted to answer this question by isolating the two possible causes and allowing each to act separately.

To test suggestion (*a*) a long cover-glass was supported over a slide by wax feet placed beneath its four corners. Some filtered suspension of *Bodo* was then let in under the cover-glass from one end, so as not to fill completely the space between the cover-glass

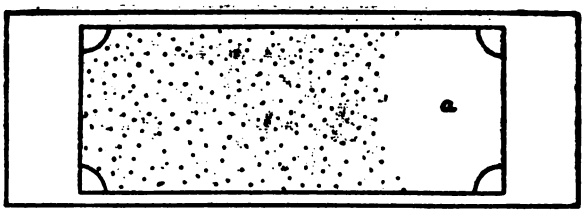

FIG. 9. Even distribution of flagellates beneath a long cover-glass unaffected by the introduction of carbonic acid at one end. *a*, carbonic acid.

and the slide. Immediately afterward from the other end of the cover-glass some water which had been saturated with carbon dioxide was let in (Fig. 9). If such a solution had an attractive influence on *Bodo*, the flagellates would have collected in the region where the two liquids merged. This they did not do.

To test suggestion (*b*) a similar preparation was made but in place of carbonic acid, reduced indigocarmine was introduced beneath one end of the cover-slip. Indigocarmine (sodium sulfindigoate) was reduced in the absence of oxygen (in a stoppered bottle) by a solution of 1 per cent glucose containing 1 per cent caustic potash. By this means the yellow leuco base is formed. As much of a concentrated solution of indigocarmine was used as the glucose would turn from blue to yellow in 1 hour in the stoppered bottle. In the presence of oxygen the yellow leuco base reoxidizes instantane-

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ously to blue indigocarmine. This takes place either when the leucobase is exposed to the air or when it comes into contact with water containing oxygen in solution. In our experiment under the cover-glass the dissolved oxygen was abstracted from a zone of the *Bodo* suspension bordering the drop of reduced indigocarmine. It was found that all the flagellates from the neighboring region of the suspension collected rapidly in this zone, forming there a crowded band (Fig. 10). Thus the *Bodo* are attracted into a region whence the dissolved oxygen has been removed. As control tests, indigocarmine alone, glucose alone, and caustic potash alone were substituted for the mixture of the three but in no case was there any aggregation of *Bodo*. This experiment was repeated with precisely the same result using in place of the reduced indigocarmine either

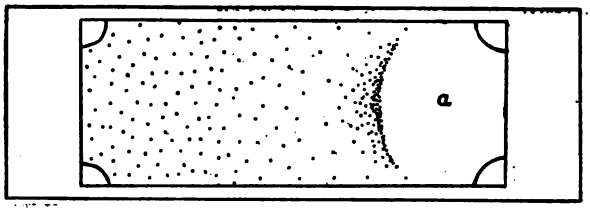

FIG. 10. Flagellates beneath a long cover-glass attracted by an oxygen absorber introduced at one end. *a*, oxygen absorber.

an alkaline solution of pyrogallic acid or of hematoxylin. Both of these solutions take up oxygen rapidly. In the case of the hematoxylin the best procedure was found to be to color the *Bodo* suspension lightly with the dye, which does not injure the flagellates, and then to introduce some of this suspension under one end of a long, supported cover-slip. Under the other end 0.1 N NaOH was let in. In the zone where the two liquids meet, the hematoxylin rapidly oxidizes in the presence of the alkali, abstracting oxygen from solution in the water. The flagellates in the neighboring region collected in a crowded band in this zone of reduced oxygen concentration. Controls in which the alkali was replaced by water and in which the hematoxylin was omitted gave no aggregation of flagellates.

In this way it can be demonstrated that the flagellates will collect into a region where the concentration of dissolved oxygen is reduced

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but that they will not react to a region of greater hydrogen ion concentration caused by dissolved carbon dioxide. The aggregation then in the center of the liquid beneath a cover-slip must be due to the attractive influence of this central region where the respiratory activity of the organisms has reduced the amount of dissolved oxygen.

The second question—why does an aggregation become a ring surrounding an ever growing clear area—has now to be answered. Do the *Bodo* leave the central area (*a*) because the concentration of dissolved oxygen has fallen here below an optimum value for them or (*b*) because the hydrogen ion concentration has increased too much?

If (*b*) is the cause, the flagellates should leave the central area sooner—that is, they should form a ring sooner—in a preparation which was originally more acid than in one originally less acid. For in the former the critical concentration of H ions which would drive out the flagellates would be arrived at earlier. This test was made. One sample of a culture of *Bodo* was given a concentration of H ions such that when tested with rosolic acid it gave the same yellow color as did tap water saturated with carbon dioxide. A second sample from the same *Bodo* culture was given a concentration of H ions showing a pink with rosolic acid. These changes in the H ion concentrations of the two samples had no effect on the activity of the flagellates as judged under the microscope. If the cause of the formation and spreading of the band is the accumulation of carbonic acid at the center of the liquid, the occurrence must take place sooner in a preparation made from the first sample than in one made from the second. The experiment showed, however, that the band was formed and spread simultaneously in the two preparations.

It must be mentioned here that in this and all other experiments when the times of aggregation or band formation were to be compared in two preparations, the cover-glasses were supported by short pieces of thin glass rod cemented to their corners, not by wax feet. By using pieces of glass having the same thickness it was ensured that the cover-glasses were at the same height above the slides. This is necessary since the height of the cover-glass influences the rate of aggregation and the distance of the equilibrium position of the band from the edge.

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It is not carbonic acid, then, that drives out the *Bodo* from the center and we must conclude that, while the organisms collect into a region where the oxygen concentration is lower than the saturation concentration for the atmospheric partial pressure, yet when the oxygen in this region falls below a certain limiting value the organisms are forced to move away again. They remain in a band surrounding the central area of lowest oxygen concentration, this band representing the optimum concentration of oxygen for them. It lies between the central exhausted region and the outside liquid into which oxygen continually dissolves from the air to replace that used up. Further, since in the crowded band the *Bodo* consume the available oxygen more rapidly than it can be replaced from outside, the size of the central area of lowest oxygen concentration continually increases, forcing the band to approach nearer and nearer to the edges of the cover-slip.

The fact that the *Bodo* band is a zone of optimum oxygen concentration at once gives the clue to the third question originally asked—why does the advancing band cease to advance when it has reached a certain distance from the edges of the cover-glass? As the band advances towards the water-air surface at the edges of the preparation it must eventually reach a position where there is a state of equilibrium between the oxygen used up by the flagellates and that diffusing inwards from the edges. At this point the band will remain stationary.

That this inward diffusion of oxygen is really the factor which controls the distance of the band of flagellates from the edge can be shown very simply as follows. A preparation with a *Bodo* band which has become stationary is placed in a gas chamber on the stage of a microscope and oxygen is passed through the chamber. Almost immediately the flagellate band commences to retire inwards towards the center of the liquid under the cover-slip. It gets gradually less square and more circular until it forms a small ring and finally becomes one mass of flagellates at the center of the preparation. When now a stream of hydrogen is passed through the gas chamber in place of the oxygen, the band reforms and slowly but continuously increases in circumference. In the first case the oxygen dissolving in the edges of the liquid and diffusing inwards causes the position of opti-

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mum oxygen concentration for the flagellates to move inwards. In an atmosphere of hydrogen, on the other hand, the oxygen goes out of solution again around the free edges of the liquid and the position of optimum oxygen concentration for the flagellates again moves outwards.

Thus oxygen is the controlling factor in the position which the flagellates take up: they collect into regions where the concentration of dissolved oxygen is an optimum for them. The behavior of the advancing band in the presence of an air bubble now receives its explanation. The bubble acts at first like the edges of the preparation, the water immediately around the bubble being saturated with oxygen at the atmosphere partial pressure of the gas, so that the flagellates are unable to move right up to its surface. But as the flagellates are continually taking up oxygen, the amount of the latter present in the bubble gradually becomes exhausted. The flagellates are thus enabled to approach closer to the bubble until they touch its surface, where they remain for a short time until nearly all the oxygen in the bubble has gone into solution and been consumed by them. Then the oxygen concentration at the surface of the bubble falls below the optimum for the *Bodo* and they leave the region altogether.

This phenomenon can be imitated as follows. A cover-slip with wax legs is placed on a slide and some of the indigocarmine-glucose-caustic potash mixture let in beneath it with a pipette. The central area of the preparation soon becomes yellow, the indigocarmine being reduced here in the absence of oxygen. This yellow area is square with its sides parallel to the edges of the cover-glass. It is surrounded by a purple band, the region between this purple band and the edge of the liquid being blue. The purple band corresponds exactly to the flagellate band. It is the region of equilibrium between oxygen consumed at the center and oxygen diffusing in from the edge. Any air bubble in the yellow area is surrounded at first by a narrow blue ring, separated from the yellow by a circular purple band. The purple band approaches slowly but continuously to the bubble until it lies on the surface of the latter and then it gradually disappears. This occurs when all the oxygen of the bubble has gone into solution and been used up. We have here an exact parallel to the behavior of a *Bodo* band surrounding an air bubble.

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We must now ask, what is the cause of the gradual slow retiral of a *Bodo* band from the edges of a preparation when left over night at room temperature, of its more rapid retiral in the cold, and of its spreading out again when replaced at a higher temperature? These phenomena also can be imitated with an indigocarmine preparation such as has just been described. When the slide with its yellow central region separated from the blue border by a square purple band, the sides of which are parallel to the edges of the cover-glass, is placed in the ice chest the purple band contracts. It retires towards the center of the solution, the corners of the square becoming rounded until it assumes the form of a ring. When the preparation is now replaced at a higher temperature the purple ring expands again, becoming square as it nears the edges of the cover-glass. When left over night in a moist chamber at room temperature the purple band recedes slowly from the edges of the liquid. Now this behavior of the purple band of partially reduced indigocarmine is due to the different solubilities of oxygen at different temperatures. On the ice more oxygen goes into solution and drives the purple band inwards. When the preparation is warmed some of the oxygen goes out of solution and the purple band approaches the edges again. Left at room temperature the concentration of dissolved oxygen at the edges of the preparation gradually rises because the water was not originally saturated with oxygen, so that the band retires slowly inwards. The cause of the similar behavior of a *Bodo* band under the same conditions must be precisely the same. The band of flagellates moves to a position nearer to or further from the edge according to the lesser or greater amount of oxygen going into solution at the different temperatures. An alternative explanation is that the *Bodo* band retires inwards at a low temperature or when left for some time because under these circumstances the flagellates change their oxygen optimum. The suggestion, however, becomes very improbable in view of the parallel behavior of the indigocarmine band, the cause of which is known.

Confirmatory Facts.

There are several facts and experiments which strikingly confirm the conclusion that the concentration of dissolved oxygen is the con-

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trolling factor in the behavior of the flagellates. Of these I shall mention three. They are: (1) the effect of the original oxygen content of the *Bodo* suspension on the rate of band formation; (2) the reduction of oxyhemoglobin by *Bodo*; and (3) the effect of a green plant in sunlight on the band.

To show the effect of the oxygen content of the suspension of flagellates on the rate of aggregation and band formation two preparations were made on slides in the manner already described. In the first preparation the liquid was let in beneath the supported cover-glass immediately after having been pipetted out of the culture jar. For the second preparation a pipetteful of liquid was taken from the same culture jar, placed in a petri dish, and exposed to the air for a short time before being let in under the cover-glass. A *Bodo* culture always contains less oxygen in solution than plain water in a similar jar at the same temperature. This is of course due to the fact that the flagellates are continually absorbing oxygen in respiration. Consequently the suspension of *Bodo* which had been exposed to the air in the petri dish had acquired a greater oxygen content than that taken directly from the culture jar. It was found that the aggregation of flagellates was much slower in the aerated than in the non-aerated preparation and that the central clear area became established later in the former.

It was to be expected that the central clear area bordered by the band of flagellates would become established later in the aerated preparation. Here it must take longer for the organisms to consume sufficient oxygen to make the central region untenable for them. But it is not at first obvious why the central aggregation should form later in the aerated preparation. It would be expected, rather, that when in the presence of more oxygen than the optimum the flagellates would migrate from any region of higher into any region of lower oxygen concentration. Now a region of *relatively* lower oxygen concentration due to the respiration of the flagellates must arise as soon in the center of the aerated as of the non-aerated preparation; nevertheless, in the former the organisms are not attracted so soon towards the center as they are in the latter. It seems thus that when the liquid is well aerated the flagellates are not sensible to a region of lower oxygen concentration: it is not until the oxygen content has been

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reduced by a certain amount throughout the slide that the *Bodo* feel the attraction of the central region. They are thus only sensible to this influence when in the presence of an oxygen concentration closely approaching the optimum for them.

This was confirmed by an experiment with an oxygen absorber such as reduced indigocarmine or pyrogallic acid. Two slides were prepared with long cover-slips supported by pieces of glass rod. Under one cover-slip was run in some *Bodo* suspension taken straight from the culture jar and under the other some of the same culture which had been aerated by exposure in a petri dish. The liquid was not allowed in either case completely to fill the place beneath the cover-slip, but a space was left at one end into which the oxygen absorber was to be run. When this had been done, it was seen that the flagellates in the aerated preparation collected much later into the region of reduced oxygen concentration next to the indigocarmine than they did in the non-aerated preparation. In fact whereas in the non-aerated liquid the *Bodo* moved at once into the region next the oxygen absorber, in the aerated liquid they did not do this until by their own respiration they had reduced the oxygen content of the general suspension to a point approaching the optimum for them. The flagellates thus gave no response to lowered oxygen concentration when in the presence of an amount of oxygen much above their optimum.

It was mentioned at the commencement of this account that in a normal cover-slip preparation the *Bodo* ring may become established in 2 minutes or it may take 2 hours to form. A series of experiments demonstrated that this great difference in time is due to different initial oxygen contents of the suspensions. If the suspension to be used is exposed to the air for some time during the preparatory filtering operation it will have a higher oxygen content than liquid taken straight from the culture jar, for the oxygen concentration in the cultures is always considerably lower than the saturation concentration under atmospheric partial pressure, and therefore in the filtering through bolting-silk oxygen is absorbed from the air. Aggregation and ring formation always take place later in a suspension that has been exposed to the air than in one that has not.

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The following is an account of four experiments which illustrate this point. The same *Bodo* culture was used throughout. In each experiment two preparations were made; (*a*) with *Bodo* suspension taken straight from the culture jar, and (*b*) with a suspension previously aerated. In Experiment 1 the aeration was done by pouring from one watch-glass to another for 15 seconds. The times taken for the band of flagellates to take up its stationary position were; (*a*) 11 minutes, and (*b*) 45 minutes. In Experiment 2 the aeration was done in the same way and the times were; (*a*) 4 minutes, and (*b*) 46 minutes. In Experiment 3 the aeration was performed by leaving a small quantity of the liquid in a watch-glass for 56 minutes at 18°C., the laboratory temperature being 23°C. The times were; (*a*) 2 minutes, and (*b*) 56 minutes. In Experiment 4 for the preparation of Slide (*a*) 10 cc. of *Bodo* suspension were kept in a watch-glass for ½ hour at 26° and for (*b*) 10 cc. were similarly treated at 17°. There was no noticeable difference in activity of the flagellates at the two temperatures. As soon as the liquids were pipetted under the cover-glasses their temperatures became identical and equal to that of the laboratory so that the different times taken for the bands to become established must have been due to the different quantities of oxygen which had gone into solution at the two temperatures. The times were; (*a*) 2½ minutes, and (*b*) 14 minutes.

The consumption of oxygen in the center of developing *Bodo* rings can be demonstrated in preparations containing hemoglobin. For this purpose a pipetteful of liquid from a *Bodo* culture is placed in a watch-glass and one or two drops of blood from a pricked finger are mixed with it. The blood becomes laked. It in no way interferes with the activity of the flagellates. If now a preparation is made in the usual way beneath a supported cover-slip, no sooner does the central aggregation of flagellates become a ring than the bluish color of the region surrounded by the ring shows that it contains reduced hemoglobin. The blue color here is in marked contrast to the scarlet of the oxyhemoglobin outside the ring of flagellates, and the distribution of hemoglobin and oxyhemoglobin is easily verified with the microspectroscope. Thus after the flagellates have used up the available free oxygen dissolved in the water, they extract that which is bound in the oxyhemoglobin. When this too is exhausted at the

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center of the slide, a circular band of flagellates is formed surrounding a region of reduced hemoglobin. As would be expected, aggregation and band formation take place much more slowly in the presence of oxyhemoglobin than in plain water for much more oxygen is available.

In a typical case two preparations were made simultaneously from the same culture; (*a*) without blood, and (*b*) with blood. During the mixing of (*b*) with blood, (*a*) was exposed to the air in exactly the same way as (*b*). The times for the ring to become established in its stationary position were; (*a*) 1 hour, 15 minutes, and (*b*) 3 hours, 40 minutes.

In the preparations containing hemoglobin the circular *Bodo* zone surrounding the central clear area, when once formed, grows in the usual way, becomes square, and takes up its stationary position some

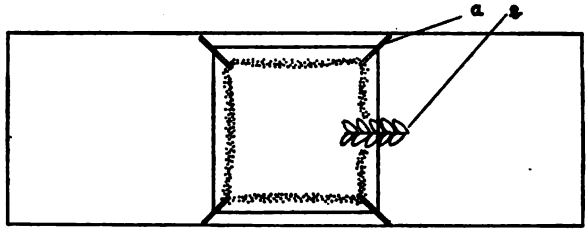

FIG. 11. Preparation kept in darkness with flagellate band in equilibrium position interrupted by a piece of moss. *a*, glass rod supporting cover-glass; *b*, moss.

distance inside the edge of the cover-slip. The whole central area within the band contains reduced hemoglobin while the edges outside it show the bright scarlet of oxyhemoglobin. The flagellate band itself lies just within the region of reduced hemoglobin.

A demonstration of the effect of oxygen on the equilibrium position of the *Bodo* band can be arranged as follows. Some suspension of *Bodo* is let in with a pipette under a cover-glass supported at its corners, and a piece of some aquatic green plant, such as a frond of moss, is pushed into the liquid from the middle of one side of the cover-glass. The slide is placed in the dark and when the band of flagellates becomes established in its stationary position it will cut straight across the moss (Fig. 11). The preparation is now exposed to diffuse sunlight, so that oxygen is produced by the plant in photosynthesis.

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The edges of the flagellate band which touched the moss on either side immediately move back from it and bend inwards (Fig. 12) and in a few minutes the band has reformed with an indentation to include the moss (Fig. 13). Replaced in the dark the band straightens

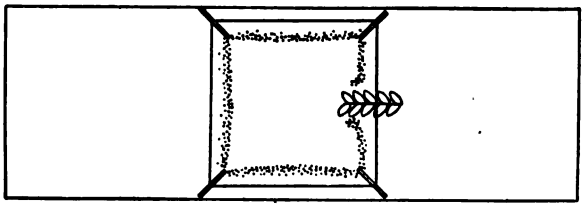

FIG. 12. Bending inward of the ends of the flagellate band in contact with the moss when the preparation shown in Fig. 11 is brought from darkness into daylight.

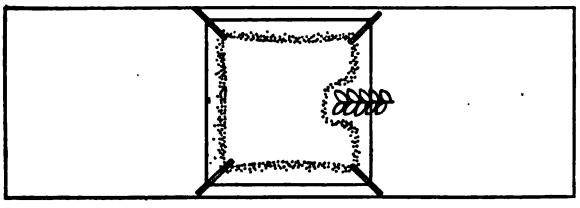

FIG. 13. Reformation of the flagellate band inside the moss after the preparation shown in Figs. 11 and 12 has been in daylight for a short time.

out again to its original position as the extra oxygen which was produced by the plant in the light is consumed by the flagellates. The two ends of the band in touch with either side of the moss bend slightly outwards towards the edge of the slide (Fig. 11) because the moss is now absorbing oxygen.

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AN INVESTIGATION INTO THE CAUSE OF THE SPONTANEOUS AGGREGATION OF FLAGELLATES AND INTO THE REACTIONS OF FLAGELLATES TO DISSOLVED OXYGEN.

PART II.

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(Received for publication, September 21, 1920.)

Previous Work.

As far as I am aware, the only workers who have attempted to find the causes of the spontaneous aggregation of flagellates are Jennings and Moore.[1] They used a flagellate called *Chilomonas paramecium* and came to the conclusion that the organisms are attracted by the carbonic acid produced by themselves. Where by chance the flagellates are more crowded together in the liquid on a slide, more carbonic acid is produced than elsewhere and in consequence yet more flagellates are attracted into these regions. The experimental evidence on which they based their conclusion was as follows. They found: (1) that when a dilute mineral acid was let in under the cover-slip by means of a capillary pipette into a suspension of the flagellates the latter were attracted by the drop; and (2) when a bubble of carbon dioxide was introduced in the same manner the organisms crowded around it, whereas they did not collect around an air bubble. Therefore, Jennings and Moore concluded, in the formation of spontaneous collections the flagellates are attracted to regions which become more acid than the remainder of the liquid, through the accumulation of the carbonic acid produced by the individuals already there.

[1] Jennings, H. S., and Moore, E. M., Studies on reactions to stimuli in unicellular organisms, VIII. On the reactions of Infusoria to carbonic and other acids, with especial reference to the causes of the gatherings spontaneously formed, *Am. J. Physiol.*, 1902, vi, 233.

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I have found that both the experimental data of these workers are correct, but that nevertheless they do not lead to their conclusion. I tried the effect of mineral acids on *Bodo* by letting the acid in under one end of a long, supported cover-slip, under which some *Bodo* suspension had just previously been introduced. In a zone parallel to the junction of the two liquids a band of flagellates rapidly accumulated. A series of such tests was made with acids of decreasing strengths and it was found that the reaction became feebler each time until a point was reached (at 0.0005 N HCl) when the *Bodo* no longer reacted to the acid at all. Nevertheless 0.0005 N HCl, tested with Congo red as indicator, is a considerably stronger acid than a saturated solution of CO_2 in water. Congo red is turned violet by 0.0005 N HCl whereas the scarlet color of this indicator in distilled water takes on a very slight bluish tinge only when the solution is saturated with CO_2.

Further it has already been shown (page 489) that the flagellates do not collect in the region next a drop of water saturated with CO_2 let in under the cover-slip. Jennings and Moore made no attempt to determine quantitatively the exact strength of acid solution to which these organisms react. In fact, we are dealing here with a phenomenon quite unconnected with spontaneous aggregation, a reaction of the flagellates to acids and the weakest effective acid is stronger than carbonic acid.

It is worthy of remark that the accumulation of a crowded zone of *Bodo* in the region in front of an acid introduced beneath the cover-slip is much more marked in aerated than in non-aerated suspensions, which is the reverse of the reaction of the flagellates to a region of reduced oxygen concentration. Indeed an acid as weak as 0.001 N HCl produces a slight collection in an aerated preparation only and none in one made by taking liquid straight from the *Bodo* culture jar. The crowded zone next the acid always dissipates before the subsequent commencement of aggregation and band formation of the flagellates in the region of low oxygen concentration.

With regard to the gas bubbles, it is true that flagellates collect around a carbon dioxide bubble and not around an air bubble, but it is equally true that flagellates collect around a hydrogen bubble. Jennings and Moore did not try the hydrogen bubble. It is evidently

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to reduced oxygen concentration that the organisms are attracted both in the case of the carbon dioxide bubble and of the hydrogen bubble. Part of the oxygen in solution in the water diffuses into the gas bubble, where the partial pressure of oxygen is nil, and so a region of reduced oxygen concentration is established in the water around the bubble. The flagellates are attracted into this region just as they are attracted into a central region under the cover-glass where the oxygen concentration is lowered by their own respiration. Just as in the latter case the aggregation occurs sooner in a non-aerated than in an aerated preparation, so the response to a hydrogen bubble is found to be greater in a non-aerated suspension.

The flagellates collect immediately around a hydrogen bubble but the intensity of the aggregation diminishes with time, until after a certain interval there are no more *Bodo* around the bubble than in the general suspension. This is because the bubble has now absorbed from the water enough oxygen to be in equilibrium with the dissolved oxygen and there is no longer a region of low oxygen concentration in the water around the bubble. After this the continually decreasing oxygen tension in the water, due to the respiration of the flagellates, falls below that in the bubble and the flagellates move away from the latter which is now giving back oxygen to the water. The center of the preparation is then cleared of *Bodo*, the peripheral band formed in the usual way, and in this process the bubble acts as an air bubble, becoming surrounded by an inner ring of flagellates which gradually approaches it.

Thus the experiments with acids and with gas bubbles not only dispose of the view that spontaneous aggregations are due to positive chemotropism to acid produced by the respiration of the flagellates but they also strengthen the explanation of the phenomenon given above.

The Effects of Excess and of Absence of Oxygen.

There is a relation between the oxygen content of the *Bodo* culture media and the preference shown by the flagellates for an optimum oxygen concentration lower than the saturation concentration of the gas in water in contact with air. For a series of determinations showed that, in the grass infusions contained in upright unstoppered

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glass jars in which the *Bodo* swarmed, the oxygen content was considerably lower than that in a similar vessel containing an equal volume of tap water.

A number of experiments was made to test the effects, if any, of excess and of absence of dissolved oxygen on *Bodo*. An ordinary cover-slip preparation was made and at the same time an equal amount of *Bodo* suspension was placed on a hollow-ground slide and exposed to the air in a moist chamber. 24 hours afterwards the usual square-shaped band of flagellates was present under the cover-glass, in the zone of optimum oxygen concentration, while on the uncovered slide the flagellates were evenly distributed throughout the drop. In spite of the oxygen concentration being above the optimum in the second preparation, the swimming activity of the individuals was alike in both. The fact that a large excess of oxygen has no effect either was demonstrated at the conclusion of a gas chamber experiment such as that described on page 492. A preparation with an established *Bodo* band was placed in the chamber and oxygen passed through the latter. The square band retired towards the center becoming a ring and then a single mass in the middle after which the flagellates dissipated to become evenly distributed through the slide. This dissipation always occurs when the oxygen concentration is more than a certain amount above the optimum; it was seen also in preparations left standing for some time (page 487). In the present instance the oxygen concentration must have been very greatly above the optimum since the gas chamber contained pure oxygen. The chamber was now closed and the preparation left over night in the oxygen atmosphere. 17 hours after the commencement of the experiment the flagellates were swimming in full activity. The preparation was then removed from the gas chamber and exposed to the air. In 3 hours and 10 minutes two aggregations had formed towards the center and in 10 hours and 10 minutes the square band was reestablished. Thus 17 hours in the presence of an oxygen concentration higher than they could ever encounter in nature had no inhibitory effect on the activity of the flagellates.

To test the effect of the complete absence of oxygen an experiment was devised as follows. An ordinary preparation was made of *Bodo* suspension beneath a supported cover-slip. This was then closed

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along the four sides with vaseline so that the suspension was everywhere shut off from the air (Fig. 14). For comparison a second preparation was made which was vaselined along two of its four open edges. This was done to eliminate any possible injurious effect of the vaseline on the *Bodo*, which would now be the same on both slides. 24 hours afterwards almost all the flagellates in the sealed preparation were motionless, just a few swimming feebly or vibrating. In the open preparation there were normal bands of flagellates at a

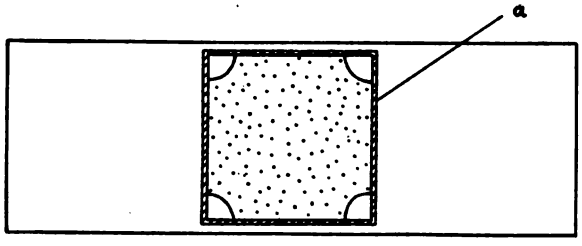

FIG. 14. Experiment to test the effect of the absence of oxygen on flagellates. Edges of the cover-glass were sealed with vaseline. *a*, vaseline.

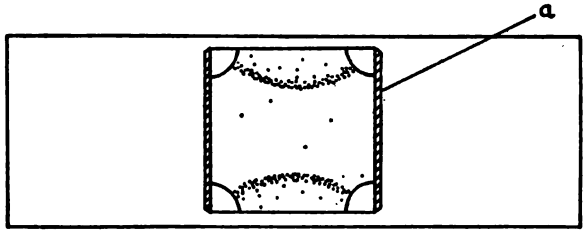

FIG. 15. Experiment to test the effect of the absence of oxygen on flagellates. Two sides of the cover-glass were sealed with vaseline. Distribution of flagellates after 24 hours. *a*, vaseline.

certain distance inside the free edges (Fig. 15). 48 hours from the start of the experiment the *Bodo* in the sealed slide were in the same motionless condition as on the previous day, while those in the open preparation were in full activity, the bands having retracted in the usual manner to form a ring near the center. A small aperture was now made in the vaseline wall surrounding the sealed preparation so that the air came into contact with the water of the suspension at this point. Instantaneously all the *Bodo* in the neighborhood of

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the aperture commenced to swim actively and arranged themselves in a band at a certain distance inside the aperture (Fig. 16). This semicircular band gradually retreated inwards as the atmospheric oxygen diffused in. The effect of the absence of oxygen is thus not immediately injurious; it stills the motion of the flagellates but after 48 hours in the absence of oxygen they recover their full activity as soon as they have access to oxygen again.

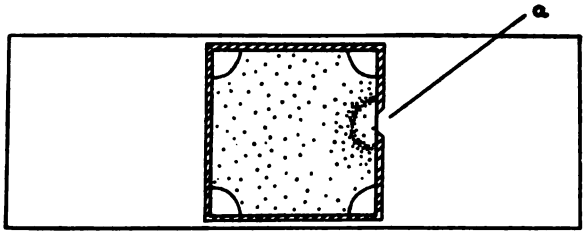

FIG. 16. Experiment to test the effect of the absence of oxygen on flagellates. Distribution of the flagellates in the preparation already shown in Fig. 14, when a hole was made in the vaseline wall at the end of 48 hours. *a*, aperture in vaseline.

Minor Phenomena of Aggregation and Band Formation.

There are several minor phenomena in the process of aggregation and band formation which have not been mentioned up to the present but which must now be described.

1. It should be added to the general description of aggregation and band formation that when very few flagellates are present in the liquid no central aggregation is formed, but after a certain time the flagellates simply leave the central region of the preparation and arrange themselves directly in the final stationary position of the band.

2. A vertical section of the *Bodo* band is not an upright wall but has the shape shown in Fig. 17. The upper part of the band is nearer to the center of the preparation than the lower part. Further, there are more flagellates present in the upper and lower regions, that is next the cover-glass and next the slide, than in the intermediate region. The latter is curved in section, the concavity being towards the edge of the preparation. The band assumes this shape as soon as it forms and keeps the same shape when in its final station-

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ary position. The relative density of flagellates in the upper and lower parts of the band varies in different preparations, being sometimes more and sometimes less marked. The degree of slope of the section of the band also varies, being sometimes more and sometimes less steep. The consequence of the greater concentration of *Bodo* at top and bottom of the band and of the slope of the latter is that when the preparation is viewed from above the band appears to be double (Fig. 18). The outer and inner lines of this double band naturally appear the further separated from one another the higher the cover-glass is placed above the slide.

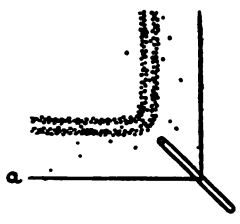

FIG. 18.

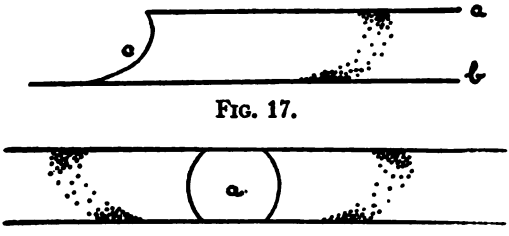

FIG. 17.

FIG. 19.

FIG. 17. Vertical section of a flagellate band. *a*, cover-glass; *b*, slide; *c*, water-air surface.

FIG. 18. Enlarged view of one corner of a square flagellate band. *a*, edge of cover-glass.

FIG. 19. Vertical section through an air bubble and a ring of flagellates surrounding it. *a*, air bubble.

The section of the band has a similar shape to that of the water-air surface at the edge of the cover-slip. This is shown in Fig. 17. It was thought at first that the form of the water-air surface might determine the form of the band, but this is not the case for a flagellate ring surrounding an air-bubble has the same sectional shape as the main band whereas the section of the water-air surface bordering the bubble is a symmetrical curve as shown in Fig. 19. The reason for the peculiar shape of the *Bodo* band remains undetermined. It is unknown why those individual flagellates which are negatively geotropic seem to have a lower optimum oxygen concentration than those which are positively geotropic. The possibility naturally arose that there were two species of *Bodo* present or two different stages in

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the life history of *Bodo sulcatus*. To settle this, the flagellates were fixed after they had formed a band by introducing the fixative beneath the cover-slip through a capillary tube previously placed in position. Fixed in this way many individuals remained in position attached to the glass so that the cover-slip could be lifted off the slide and then the upper and lower flagellates stained and mounted separately. The flagellates attached to the cover-glass were found to be identical with those on the slide. We are thus dealing with a single species but the possibility remains that the positively and negatively geotropic individuals are in different stages of their life cycle.

3. A further point that has not yet been mentioned is the following. When an oxygen absorber such as reduced indigocarmine is introduced beneath a cover-slip next to a suspension of *Bodo*, a band of flagellates collects in a zone bordering the oxygen absorber in the manner already described. After this principal band has formed a subsidiary band containing relatively very few individuals makes its appearance nearer in to the oxygen absorber than the principal band. The flagellates in this inner band are close up beneath the cover-glass and move very sluggishly in striking contrast to the intense activity of those in the principal band. When the latter moves away to take up its definitive position just inside the edges of the cover-slip, the inner band remains for some time in place and then gradually the individuals composing it dissipate to join the principal band.

The reason for this inner band may be that a few individuals have a lower oxygen optimum than the rest. This explanation seems unlikely, however, because the inner band is not a permanent one; after a certain time it dissipates. More probably some individuals chance to swim into the region of lowest oxygen concentration and are trapped there because, as was shown on page 504, the effect of absence of oxygen is to inhibit swimming activity. This suggestion is supported by the fact that in ordinary spontaneous band formation an inner band is best developed in those preparations in which the central region is cleared most rapidly. Here more individuals would be left behind, trapped in the region of least oxygen.

4. There are of course many kinds of bacteria present in the *Bodo* cultures. In the preparations on slides many of the bacteria arrange themselves in zones of optimum oxygen concentration in the manner

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originally described by Beyerinck.[2] The bacteria, however, do not commence by forming obvious central aggregations which then become converted into rings. This is, perhaps, because they are less motile than the flagellates: they collect more slowly than the latter into their zones of optimum oxygen.

Now when a bacterial optimum position coincides with that of *Bodo*, there is frequently an antagonism between the two organisms, the bacteria preventing the flagellates from taking up their normal position. In particular, there were frequently present certain cocci the optimum position for which was situated at the middle-depth of the *Bodo* band and certain bacilli whose optimum coincided with the lower and outer portion of the *Bodo* band. When a preparation was made from a culture in which these bacteria were present in quantity the *Bodo* were excluded from the middle and lower parts of their zone and all forced to occupy the upper and inner part. The bacteria took up their position more slowly than the flagellates, consequently when the band first formed the flagellates occupied both upper and lower regions, as shown in Fig. 17, but when the bacteria arrived they drove the flagellates out of the lower region. When a preparation having a *Bodo* band with these bacteria in its lower part and a piece of moss cutting across the band as described on page 498 is removed from darkness to light, all the flagellates and bacteria move inwards to take up a new position, as shown in Figs. 11, 12, and 13 (Part I). But the *Bodo* move back much more rapidly than the bacteria and, arriving first at the new position of optimum oxygen, are able to place themselves in a band extending in depth from cover-glass to slide. Later on the bacteria arrive and drive out the flagellates from the lower and middle parts of their zone.

SUMMARY.

Spontaneous aggregations of flagellates are formed under the cover-glass because the organisms are attracted to and remain in regions where the concentration of dissolved oxygen is less than the saturation concentration under atmospheric partial pressure. These regions of

[2] Beyerinck, M. W., Über Atmungsfiguren beweglicher Bakterien, *Centr. Bakt.*, 1893, xiv, 827.

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lessened oxygen content arise towards the center of the liquid beneath the cover-glass, owing to the oxygen consumed by the flagellates in respiration not being replaced here by the solution of atmospheric oxygen, as it is along the edges of the liquid. The flagellates, however, are insensitive to the attraction of regions of lessened oxygen concentration when the oxygen concentration throughout the liquid is above a certain value. Therefore, for the aggregations to form, either the initial concentration of dissolved oxygen must be below this limiting value, or an interval of time must first elapse after the making of the preparation until the respiration of the organisms has reduced the oxygen concentration throughout the liquid down to this limiting value. The aggregations will then form because the flagellates have become positively chemotropic to the lower concentration of oxygen at the center of the liquid.

Once established, such an aggregation of flagellates does not remain long in the same form. An area free from flagellates appears at the center of the aggregation so that the organisms lie in a circular band surrounding the clear area. The latter increases in size and its bordering band of flagellates in diameter, the band gradually becoming less circular and more square in shape, if the cover-glass is a square one. The clear central area is a region where the oxygen consumption of the flagellates has reduced the oxygen content to such a low value that the organisms are forced to leave the region. They collect in a band where the concentration of dissolved oxygen is an optimum for them. It is the equilibrium position between the oxygen consumed at the center and that diffusing in from the edges of the liquid. As the consumption at the center is more rapid than the replacement from the edge, the flagellate band moves outwards until it becomes stationary at a position where the rates of consumption and replacement of oxygen are equal.

Although the flagellates collect in this manner in regions of optimum oxygen concentration, yet greater concentrations of dissolved oxygen have no injurious effect on them. Concentrations of dissolved oxygen lower than the optimum have the effect of inhibiting the movement of the flagellates. They recover their activity, however, immediately they are given access to dissolved oxygen again.

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Work done in the past on chemotropism of flagellates will have to be revised in the light of the above facts, since the oxygen content of solutions used has never been taken into account.

The work was done during the months of June to September, 1919, at the Laboratory of the Marine Biological Association, Plymouth. I wish to thank the Director and Staff for the help and advice given to me. I wish also to thank the Trustees of the Ray Lankester Fund for having nominated me to an Investigatorship. Mr. C. Clifford Dobell F.R.S. was kind enough to identify the species and examine fixed preparations for me.

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THE EQUILIBRIUM BETWEEN HEMOLYTIC SENSITIZER AND RED BLOOD CELLS IN RELATION TO THE HYDROGEN ION CONCENTRATION.

By CALVIN B. COULTER.*

(From the Hoagland Laboratory, Brooklyn.)

(Received for publication, December 22, 1920.)

INTRODUCTION.

The reversible nature of the union between various kinds of antigen and antibody in the usual physiological saline solution has been demonstrated many times.[1-12] Landsteiner and Jagić[3] have interpreted the reaction between antigen and antibody as a reversible reaction which is essentially similar to that taking place between simpler and definitely known chemical substances and in which an equilibrium is reached depending upon the concentration of the reacting substances and the temperature. Further evidence that suggests such an equilibrium state is given by Bail[9] and Matsui[11] in experiments on the splitting off of anticholera sensitizer. However, this point cannot be regarded as definitely proved by experiment.

That the chemical reaction, or the acidity or alkalinity, is a factor in the combination of antibody and antigen appears in any event

* Van Cott Fellow in Pathology.

[1] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905.

[2] Morgenroth, J., *Münch. med. Woch.*, 1903, l, 61.

[3] Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1903, l, 764.

[4] Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1904, li, 1185.

[5] Landsteiner, K., and Reich, M., *Centr. Bakt., 1te Abt., Orig.*, 1905, xxxix, 83, 712.

[6] Bail, O., and Tsuda, K., *Z. Immunitätsforsch., Orig.*, 1908-09, i, 546.

[7] Tsuda, K., *Z. Immunitätsforsch., Orig.*, 1909, ii, 225.

[8] Spät, W., *Z. Immunitätsforsch., Orig.*, 1910, vii, 712.

[9] Bail, O., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 202.

[10] Bail, O., and Rotky, K., *Z. Immunitätsforsch., Orig.*, 1913, xvii, 566.

[11] Matsui, I., *Z. Immunitätsforsch., Orig.*, 1915, xxiii, 233.

[12] von Liebermann, L., *Biochem Z.*, 1907, iv, 25; *Arch. Hyg.*, 1907, lxii, 277.

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from a number of investigations. Von Liebermann[12] found that small amounts of alkali inhibit the action of hemolytic sera, while acid in small amount increases this action and in larger amount inhibits it. Hecker,[13] Sachs and Altmann,[14] and von Eisler[15] have confirmed this effect of alkali. Rondoni[16] has carried the analysis of this phenomenon farther and has shown the action of alkali in inhibiting the union of hemolytic sensitizer and red cells, and a similar but less marked effect of acid, which did not depend apparently upon destruction of the immune substance but rather upon a reversible modification of it.

The influence of reaction has been shown likewise on the dissociation or splitting off of antibody from combination with its antigen. Hahn and Trommsdorf[17] were able to separate agglutinin from sensitized bacteria by digestion with N/100 NaOH and almost as well with N/100 H_2SO_4, while physiological saline solution was found ineffective. Von Liebermann and von Fenyvessy[18] effected the separation of hemolytic sensitizer from sensitized cells by dilute H_2SO_4. Rondoni[16] found that digestion of sensitized cells with alkali or acid yielded a larger amount of free sensitizer than did a like volume of physiological saline solution. The separation in alkali was more complete than in acid; in several experiments with alkali approximately 60 per cent of the total sensitizer in combination was obtained free.

The presence of electrolytes appears also to be concerned in this combination. Although Ferrata[19] and Sachs and Teruuchi[20] have shown that sensitizer and cells will combine in a salt-free medium, von Eisler[21] found that the combination is less rapid and complete in salt-free than in salt-containing media. Kosakai[22] has found

[13] Hecker, R., *Arb. Inst. Exp. Therap. Frankfort*, 1907, iii, 39.

[14] Sachs, H., and Altmann, K., *Berl. klin. Woch.*, 1908, xlv, 699.

[15] von Eisler, M., *Centr. Bakt., 1te Abt., Orig.*, 1908, xlvi, 353.

[16] Rondoni, P., *Z. Immunitätsforsch., Orig.*, 1910, vii, 515.

[17] Hahn, M., and Trommsdorf, R., *Münch. med. Woch.*, 1900, xlvii, 413.

[18] von Liebermann, L., and von Fenyvessy, B., *Centr. Bakt., 1te Abt., Orig.*, 1908, xlvii, 274.

[19] Ferrata, A., *Berl. klin. Woch.*, 1907, xliv, 366.

[20] Sachs, H., and Teruuchi, Y., *Berl. klin. Woch.*, 1907, xliv, 467, 520, 602.

[21] von Eisler, M., *Z. Immunitätsforsch., Orig.*, 1909, ii, 159.

[22] Kosakai, M., *J. Immunol.*, 1918, iii, 109.

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extraction with isotonic saccharose solution an effective means for separating sensitizer from cells.

Michaelis and Davidsohn[23] have endeavored to relate the phenomenon of specific agglutination to the electrical properties of the reacting substances, but state that typhoid bacilli and immune serum combine readily when the particles of both are negatively charged, so that their union cannot depend upon an affinity due to opposite electrical charge. They conclude further that specific typhoid agglutination and precipitation are independent of the hydrogen ion concentration—a conclusion which if applied to the general reaction between antigen and antibody would seem at variance with the facts related above.

We have shown in an earlier paper[24] that the agglutination of sensitized sheep cells has an optimum at pH 5.3 at which point the occurrence of agglutination is independent of the presence of electrolyte. This point does not coincide with the isoelectric point of the cells which was found for both the normal and sensitized cells to be about pH 4.65, and the suggestion was made that the optimum for agglutination is related to the isoelectric point of the immune serum. The present work is an outcome of the earlier investigation and is concerned with the relation of the hydrogen ion concentration to the union of hemolytic sensitizer and cells.

EXPERIMENTAL.

The methods employed were similar to those already described. To investigate the combination of sensitizer with cells in the absence, as far as possible, of electrolyte, sheep cells were washed in four changes of isotonic saccharose solution after washing in saline solution. The cells were made up to 10 per cent by volume of the concentrated sediment in saccharose solution. A series of eight to ten tubes was prepared, each containing 5 cc. of isotonic saccharose solution (9.2 per cent) and varying amounts by drop addition of N/10 NaOH or N/10 HCl. To each tube were then added precisely 0.1 cc. of undiluted immune rabbit serum and immediately afterward

[23] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

[24] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

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5 cc. of 10 per cent cell suspension. The tubes were stoppered with paraffined corks, gently agitated, and kept in the water bath at 38°C. for 35 minutes, with gentle agitation every 5 minutes. The tubes were then centrifugated and the supernatant fluid was drawn off into two equal portions. To the first portion indicator was added and the pH determined colorimetrically by comparison with a standard series. The second portion served as a color screen for the standard tube. After the determination of the pH value, the first tube was titrated with N/100 HCl or N/100 NaOH to a pH between 6.5 and 6.0 and the amount of acid or alkali so required added to the second portion of test fluid. This portion was then diluted with saline solution and its content of sensitizer determined by titration in the usual way, with physiological saline solution as the medium. 0.04 cc. of guinea pig serum was used as complement. The cells used were 0.5 cc. of a 3 per cent suspension of the same sheep cells used in the first part of the experiment. The total volume of each tube was 2.0 cc. In consequence of the adjustment of the reaction of the test fluid and the buffer action of the complement added the hydrogen ion concentration of the tubes in this titration was sensibly constant. For each experiment the immune serum itself was titrated directly, using the same saline solution, complement, and cells. The hemolytic value was determined by interpolation, as, for instance, where with a 1:10 dilution of the supernatant test fluid the readings were 0.4 cc. complete, 0.35 cc. almost complete, 0.3 cc. ±; the value chosen was 0.375 cc. By this means and by the use of a 3 per cent cell suspension the error is within 5 per cent of the true value for any given reading. In the calculations the alteration in volume of the test fluid by the two additions of acid or alkali was taken into consideration.

The effect of electrolyte was determined by adding 1 or 2 cc. of physiological saline solution together with 4 or 3 cc. of saccharose solution to a series of tubes and then adding acid or alkali, sensitizer, and cells as before.

The dissociation of sensitizer from cells was investigated as follows: 5 cc. of concentrated cell sediment from saccharose were sensitized with approximately 50 units of sensitizer per unit of cells in a volume of 50 cc. Saccharose solution was used as the medium so that the

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only electrolyte present was that added with the immune rabbit serum. The mixture of cells and serum was left standing for 30 minutes in the water bath at 38°C. and 1 hour in the refrigerator at about 8°C., then centrifugated, and the supernatant fluid drawn off. The pH of this was usually about 6.5. The cells were then washed once or twice in saccharose solution and made up to 10 per cent strength. From this point the procedure was the same as for the combination of sensitizer except that the addition of 0.1 cc. of sensitizer was omitted. The supernatant fluids from the sensitization and

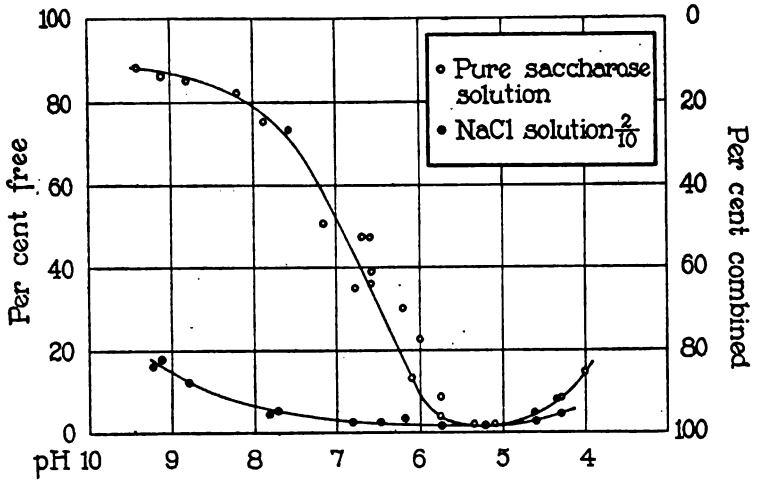

FIG. 1. Curve showing the proportion of the total sensitizer present either free or combined with cells, when the two combine *de novo*.

from the washing of the sensitized cells were titrated for their content of antibody and the sum of these values subtracted from the value of the immune serum itself. The remainder gives the amount of sensitizer actually in combination with the cells.

The results of both series of experiments are given in the form of curves, Fig. 1 for combination and Fig. 2 for dissociation. The abscissæ represent pH values, the ordinates the percentage of the total amount of sensitizer present either free or combined with cells. The curves show that near pH 5.3 the amount of sensitizer free in the supernatant fluid is at a minimum and the amount combined with the

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cells is at a maximum. On the alkaline side of this point in the absence of electrolyte the percentage of sensitizer uncombined increases with the alkalinity and reaches a maximum of nearly 100 per cent at about pH 10. On the acid side of pH 5.3 the percentage of sensitizer uncombined increases with the acidity but somewhat less rapidly than for a corresponding increase in alkalinity.

It is impossible to carry the observations to reactions more acid than pH 4 on account of hemolysis. The fragility of heavily sensitized cells is well known; both normal and sensitized sheep cells

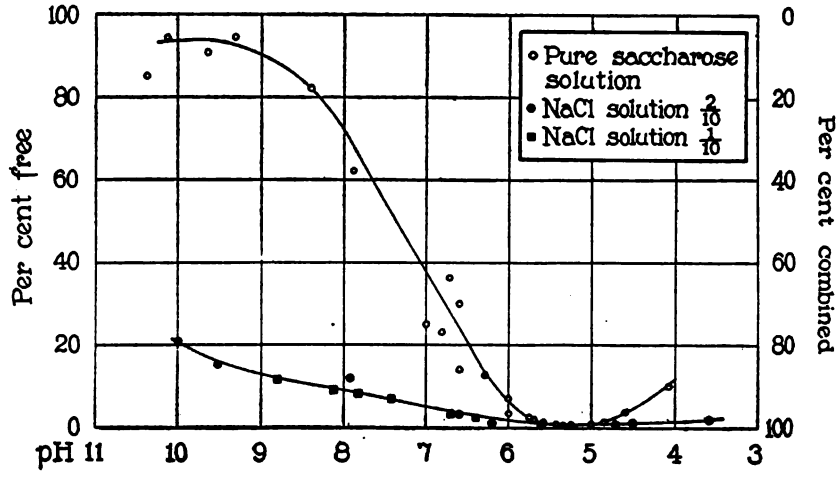

FIG. 2. Curve showing the proportion of the total sensitizer present either free or combined with cells, when the sensitizer dissociates from combination.

can endure, in the absence of electrolyte, a reaction of pH 4.5 without a trace of hemolysis provided they are not agitated. At this and more acid reactions a considerable degree of hemolysis can be caused by shaking or even gentle agitation. The agitation to which the cells were subjected while in the water bath caused a trace of hemolysis at all reactions; this hemolysis was apparently no greater at pH 10 than at pH 7.4, the normal reaction of the blood. Numerous observations were made on the acid side of pH 5 which are not recorded because of the difficulty in satisfactory colorimetric determination

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pH value in consequence of hemolysis. All agree, however, ing an increased dissociation with increased acidity.[25]

presence of electrolyte as NaCl greatly increases the proportion itizer combined with cells at all reactions except those near . At this point the combination of sensitizer with cells is ident of the presence of electrolyte. This recalls the obser- to which reference has already been made, that the agglutina- sensitized cells is independent of electrolyte at pH 5.3; it occurs ily in the presence as in the absence of salt.

a comparison of the two curves (Figs. 1 and 2) it is seen that re almost identical. Since the volumes in the experiments actically constant it is evident that, under the conditions combination *de novo* and of dissociation from combination, ilibrium is established in a given volume between the amount tizer free and that combined with cells, for any given hydrogen centration.

isoelectric point of serum globulin in which fraction the immune are believed to be carried has been given by Rona and lis[26] as about pH 5.4, and the isoelectric point of typhoid nin has been found by Michaelis and Davidsohn[23] to lie near . It is probable that the hemolytic sensitizer used here has ne value. The point of maximal combination of sensitizer ls coincides therefore with the isoelectric point of the sensitizer. amphoteric electrolytes, with which the immune bodies must sed on the basis of their behavior in the electric field (Michaelis vidsohn;[23] Landsteiner and Pauli[27]), owe their electrical charge

was found that no destruction or irreversible modification of the sensitizer ht about by the degrees of acidity or alkalinity reached in the experi- A series of tubes, each containing 0.1 cc. of sensitizer in a volume of f saccharose, was brought to various reactions corresponding to those in riments with cells, and kept at 38°C. for 35 minutes. After centrifuga- supernatant fluids were adjusted in reaction and titrated for antibody No significant differences were found between any of the tubes; the es were within the experimental error.

na, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

dsteiner, K., and Pauli, W., *25th Kong. Inn. Med.*, cited by Landsteiner, loide und Lipoide in der Immunitätslehre, in Kolle, W., and Wassermann, dbuch der Pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii,

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to ionization. On the alkaline side of the isoelectric point they ionize as acids, on the acid side as bases; at the isoelectric point the ionization is at a minimum. It is evident that the combination of sensitizer and cells is related intimately to the ionization of the immune body. The curves showing the fraction of sensitizer free in solution in a salt-free medium follow closely the curves given by Sörenson,[28] after Michaelis,[29] to represent the degree of ionization of an amphoteric electrolyte. The ionized fraction of the sensitizer, both as anion and as cation, corresponds with that fraction which is uncombined with cells, so that we may conclude that the cells combine only with the undissociated molecules of sensitizer.

The ionization of the cells appears not to be a factor in their combination with sensitizer. There is no inflection in the curves at pH 4.6, the isoelectric point of the cells. At pH 5.3, the reaction at which the maximal amount of sensitizer is combined, the cells are considerably ionized as is demonstrated in the curve showing their rate of movement in the electric field.[24]

On the alkaline side of the isoelectric point proteins combine only with cations.[30] In the presence of NaCl, a Na salt could be formed therefore with the immune body at reactions more alkaline than pH 5.3. If this salt had a small dissociation constant, so that only a small concentration of ampholyte anion could exist in the presence of Na without combining to form undissociated salt, the degree of ionization of the ampholyte would be represented by such a curve (Michaelis[31]) as that showing the proportion of sensitizer uncombined in the presence of NaCl. While we possess no information as to the degree of dissociation of such a Na sensitizer salt, the effect of NaCl is at least suggestive of a depression in the ionization of the sensitizer with combination between the cells and all undissociated molecules of sensitizer, either pure or united with cation to form a salt.

[28] Sörenson, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
[29] Michaelis, L., *Biochem. Z.*, 1911, xxxiii, 182.
[30] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.
[31] Michalis, L., *Biochem. Z.*, 1920, ciii, 225.

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CONCLUSIONS.

1. In a salt-free medium the proportion of the total amount of hemolytic sensitizer present, combined with the homologous cells, reaches a maximum of almost 100 per cent at pH 5.3. On the alkaline side of this point the proportion combined diminishes with the alkalinity and reaches a minimum of approximately 5 per cent at pH 10. On the acid side of pH 5.3 the proportion combined diminishes with the acidity but somewhat less rapidly than for a corresponding increase in alkalinity.

2. The presence of NaCl greatly increases the proportion of sensitizer combined with cells at all reactions except those in the neighborhood of pH 5.3. At this point the combination of sensitizer with cells is independent of the presence of electrolyte.

3. The curves representing the proportion of sensitizer combined or free run almost exactly parallel, both when the sensitizer combines *de novo* and when it dissociates from combination; therefore, in constant volume, at a given hydrogen ion concentration, and at a given temperature, an equilibrium exists between the amount of sensitizer free and that combined with cells.

4. The combination of sensitizer and cells is related fundamentally to the isoelectric point of the sensitizer.

5. The dissociated ions of the sensitizer, formed either by its acid or its basic dissociation, do not unite with cells. Combination takes place only between the cells and the undissociated molecules of the sensitizer.

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SUMMATION OF DISSIMILAR STIMULI APPLIED TO LEAFLETS OF SENSITIVE BRIER (SCHRANKIA).

By LEWIS B. BIBB.

First Lieutenant, Medical Corps, U. S. Army.

(Received for publication, January 15, 1921.)

The writer has made experiments upon the leaflets of *Schrankia uncinata* Willd, or sensitive brier (Fig. 1), to determine whether subminimal stimuli of different character, such as chemical and mechanical, when applied simultaneously would combine their effects so as o reinforce each other and produce a visible response. *Schrankia* ppeared suitable for this purpose because the closure of one leaflet ıechanically stimulates the next distal leaflet and causes it to close lso (this will be referred to as secondary stimulus), and thus a wave closure is started which does not stop until all distal leaflets on the me side of the mid-rib are closed. Moreover, the intensity of the condary stimuli is uniform as regards the leaflets of any given nna, but varies according to the time of day. The intensity of the ondary stimulus is insufficient in the early morning to produce a ible response, but in the late afternoon it is sufficient to provoke mpt closure of the distal leaflets in turn. It was believed there- e that by choosing different times of the day for the experiment intensity of the mechanical stimulus due to the effect of one leaflet another could be varied at will, and that, at some optimum hour, stimulating effect referred to would barely fall short of provoking sponse. When this hour was found by actual trial in each indi- ıal case, it was planned to apply subminimal stimulation and then bserve whether the closure of one leaflet was followed by the ıre of the next distal leaflet; that is, in the early morning, a single et of *Schrankia* can be caused to close against the next distal et without causing the latter to close (Fig. 2). In the late after- , on favorable days, the closure of any proximal leaflet inevitably okes closure of the next distal leaflet (Fig. 3). The problem was

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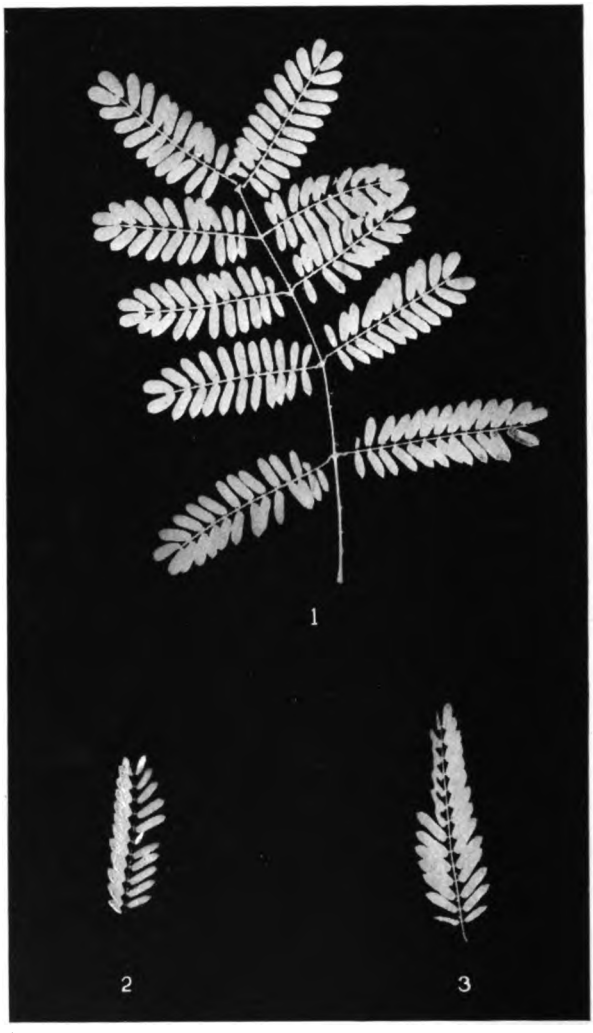

FIG. 1. Silhouette of leaf fastened (by a quick movement) between panes of glass and then fixed by heat to prevent closure of leaflets. Five pairs of pinnæ are shown, each consisting of about a dozen pairs of leaflets.

FIG. 2. Silhouette made artificially to show appearance of pinna after a single leaflet has been caused to close by stimulation. This result usually is seen in the forenoon.

FIG. 3. Silhouette made under artificial conditions to show appearance of pinna after wave of closure has involved all leaflets distal to the leaflet originally stimulated. This result is usually seen in the late afternoon.

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to ascertain whether subminimal chemical stimulation could be so applied as to lower the threshold of susceptibility to stimulation and cause the leaflets to behave in the forenoon as they normally behave in the afternoon.

Several attempts were made to transplant *Schrankia*, but such treatment interfered with its reactivity. A spray of the plant was isolated within an Erlenmeyer flask, with the object of securing a definite concentration of chemical substance (chlorine gas or ammonia fumes) in the atmosphere surrounding the leaflets; but this experiment proved a failure because of condensation of water on the inner surface of the flask. Attempts were made to summate subminimal mechanical stimuli with subminimal stimulation due to the concentration by a lens of the rays of the sun, and to summate mechanical stimuli with stimulation of different acids, but these experiments failed.

Finally, on June 22, 1919, at 11.15 a.m., the following experiment was performed. Several pinnæ of a plant growing in the open were tested by touching a more or less proximal leaflet, and in each instance, the touched leaflet closed without inducing closure of its next distal neighbor. A petri dish containing ammonia water (27 per cent) was then placed carefully underneath the adjacent compound leaf (consisting of five pairs of pinnæ) and after a few seconds, a single leaflet of each of four pinnæ was touched. In every instance, all leaflets, on the same side of the mid-rib, lying distal to the touched leaflet closed in a regular succession or wave. These results were confirmed by repeating the experiment on June 24, at 4 p.m.

On July 3, in the forenoon, several pinnæ of an individual plant were tested by touching one leaflet of each pinna. In every instance, the touched leaflet closed without causing closure of the next leaflet. A stream of chlorine gas mixed with air (10 per cent chlorine) was then allowed to flow gently over each of several pinnæ taken in turn. 6 seconds after the beginning of the application of the chlorine mixture, a proximal leaflet was touched; it closed and caused the closure of the next distal leaflet. The wave of closure proceeded until all distal leaflets on the same side of the mid-rib were closed. The fact that the leaflets on the opposite side of the mid-rib did not close was accepted as proof that the chlorine mixture did not alone cause the closure of the distal leaflets. All of several leaflets tested gave similar results.

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SUMMARY AND CONCLUSIONS.

1. The pinnæ of sensitive brier or *Schrankia* are favorable for demonstrating summation of dissimilar stimuli.

2. The demonstration was made as follows: A time of day was chosen when the closure of a single proximal leaflet did not provoke closure of the next distal leaflet. An irritating gas was applied to the pinna. A few seconds later, a single leaflet was touched; it closed, induced closure of the next distal leaflet, and inaugurated a wave of closure which proceeded until all leaflets on the same side of the mid-rib were closed.

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COMPARATIVE STUDIES ON RESPIRATION.

XV. The Effect of Bile Salts and of Saponin upon Respiration.

By MATILDA MOLDENHAUER BROOKS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, January 15, 1921.)

It was pointed out by Osterhout[1] that substances which increase the electrical conductivity of tissues are antagonized by those which decrease it. An illustration of this is seen in antagonism between NaCl (which increases conductivity) and Na taurocholate[2] (which decreases it). This case has additional interest as an example of antagonism between anions as well as of antagonism between organic and inorganic substances.

The experiments on electrical conductivity indicated that Na taurocholate acts like $CaCl_2$ in decreasing conductivity and in antagonizing NaCl. Whether this similarity would be found if other criteria were used remained an open question.

The experiments of the writer[3] showed that, when respiration is employed as a criterion, $CaCl_2$ antagonized NaCl. Similar results were obtained by Gustafson.[4] In view of this it became especially interesting to study the behavior of tissues under the influence of Na taurocholate.

In the experiments which were made for this purpose the organism used was *Bacillus subtilis* and the technique was similar to that described in previous papers.[5] The results are shown in the figures. Fig. 1 shows the effects of sodium taurocholate (0.0000125, 0.00015, 0.001, 0.0025, and 0.01 M) upon the rate of respiration (expressed as per cent of the normal rate). This organic salt is similar in its

Osterhout, W. J. V., *Science*, 1915, xli, 255.
Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 405.
Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.
Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 17.
Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5, 331.

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toxicity to lanthanum nitrate in that very dilute solutions produce pronounced effects. There is slight increase in the rate in 0.0000125 M; the respiration is normal in 0.000015 M, while in higher concentrations there is a decrease in the rate. In calculating the molecular

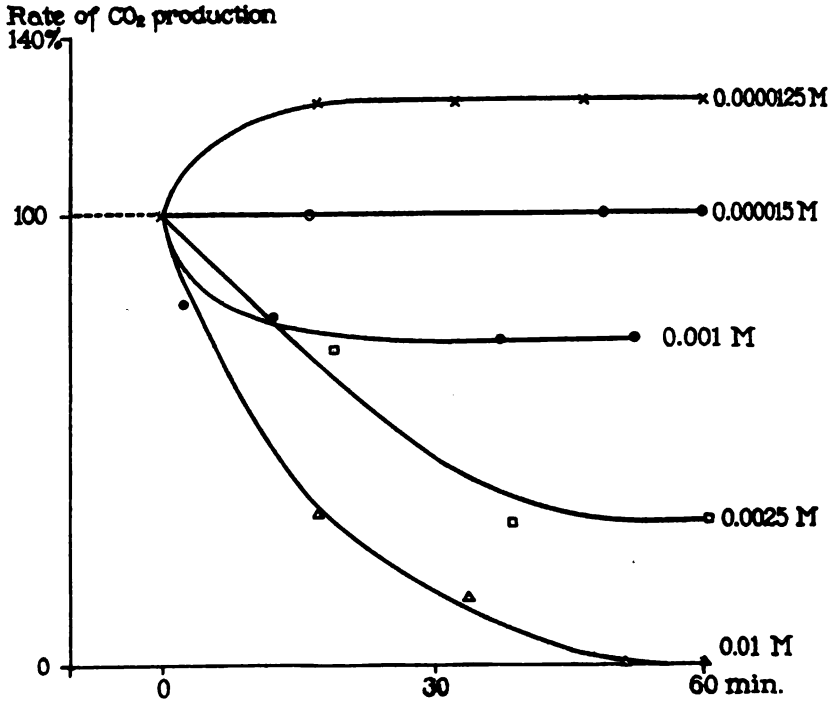

FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.0000125, 0.000015, 0.001, 0.0025, and 0.01 M Na taurocholate. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

concentrations of this salt an approximate estimate only could be made because the purity of the salt is doubtful. In making up the solutions 1 gm. of sodium taurocholate in 100 cc. of distilled water was calculated to make about 0.02 M. During the first 10 minutes the

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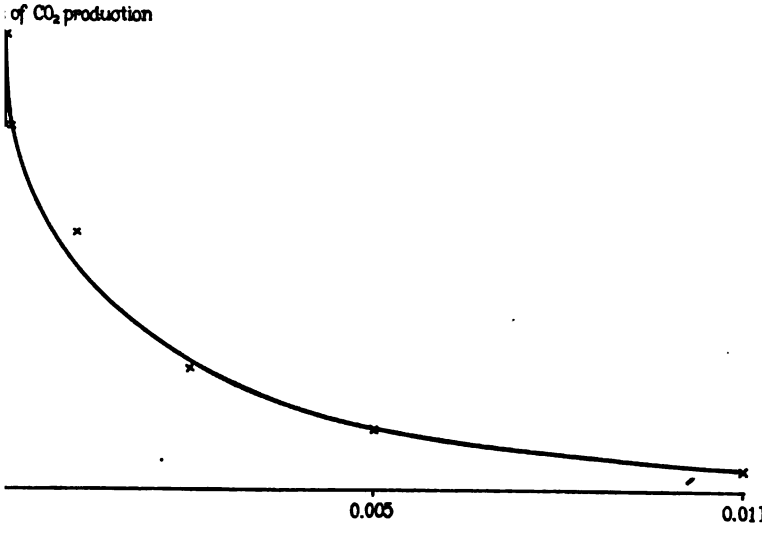
teria are under normal conditions and the curve (broken line) is izontal. After this (at the point marked 0 on the abscissa) the is added. For example, the addition of sufficient Na taurocholate nake the concentration 0.0000125 M produces a rise in the rate, ch remains constant during the period of experimentation. These ves are selected from a number of similar typical curves, and each esents one experiment.



G. 2. Curve showing the rate of respiration of *Bacillus subtilis* (expressed as ent of the normal) as affected by Na taurocholate. Normal rate as in Fig. 1. age of three experiments; probable error of the mean less than 2 per cent e mean.

g. 2 shows the effects of various concentrations of sodium tauro- ate upon the rate of respiration, expressed as per cent of the nor- rate. The rate indicated is that produced after the bacteria had in contact with the salt for 1 hour. The figure shows that sodium ocholate produces an increase in the rate at a concentration of 0125 M and a decrease in concentrations higher than 0.000125 M. of interest to note that the concentrations which are most favor- to the respiration of *Bacillus subtilis* are very dilute. The abscis- epresent the concentrations of the salts used, the ordinates repre- the rate of respiration.

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Fig. 3 shows antagonism between NaCl and Na taurocholate. In this figure the abscissæ represent the molecular proportions of each component in the mixture: thus the ordinate at the extreme left represents the rate in a pure solution of NaCl; the ordinate at the extreme right, the rate in a solution containing 95 parts of NaCl and 5 parts of Na taurocholate. In a solution containing 1 part of Na taurocholate

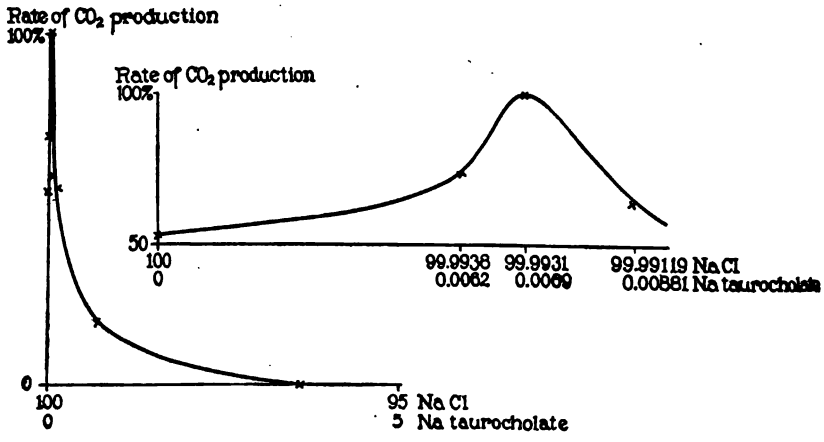

FIG. 3. Curve showing antagonism between NaCl and Na taurocholate. The ordinates represent the rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the solution used. Thus the ordinate at the extreme left represents the rate in 0.8 M NaCl while the ordinate at the extreme right (in the lower figure) represents the rate in 95 parts of NaCl and 5 parts of Na taurocholate (molecular proportions). Mixtures in which the proportion of Na taurocholate was greater showed no respiration.

Since the changes near the beginning of the curve could not be shown accurately this portion is represented on an enlarged scale in the upper part of the figure. The optimum proportion for the production of CO_2 is 99.9931 parts of NaCl and 0.0069 parts of Na taurocholate. Normal rate as in Fig. 1. Average of three experiments; probable error of the mean less than 2 per cent of the mean.

to 14,375 parts of NaCl the rate of respiration was practically normal. In all other proportions it was less than normal. For the sake of clearness, the portion of the figure at the extreme left has been drawn on an enlarged scale and is inserted above. These proportions are quite different from those found by Osterhout (500 of NaCl to 1 of Na taurocholate) to be most favorable, but the difference may be due to the

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fact that in the experiments reported by Osterhout the highest proportion of NaCl used was 500 to 1. Since this shows good antagonism, higher proportions were not tried, as the object of the experiment was merely to demonstrate antagonism and not to ascertain the most favorable mixture.

Owing to the unusual results obtained with high dilutions of Na taurocholate, it was thought advisable to procure Na taurocholate from several different sources. The original product used was Merck's. An additional sample was obtained from Eimer and Amend, and some purified bile salts were kindly sent by Dr. Morgulis and Dr. Hecht. Experiments made with all the above different samples were in remarkably good agreement, thus showing that the results were not due to the impurities which might have been present in the Na taurocholate.

Experiments on recovery were made with a mixture of NaCl and Na taurocholate (which produced a decrease in the rate of respiration). In the course of an hour, after removal of the bacteria from the mixture to 0.75 per cent dextrose, the rate did not quite attain the normal.

A few experiments with saponin were tried, inasmuch as it seemed possible that the same mechanism might be responsible for the action of Na taurocholate and for that of saponin. Eighteen concentrations of saponin were used (ranging from 0.001 to 0.0000005 M), including those which produce an increase in the rate of respiration in the case of Na taurocholate. It was found that the concentrations which were greater than 0.00005 M produced a progressive decrease in the rate, while those which were less than 0.00005 M gave the normal rate of respiration (100 per cent). None gave an increase in the rate of respiration.

Both saponin and Na taurocholate are exceedingly effective in lowering surface tension but their effects on respiration are so different as to indicate that surface tension does not play an important part in this connection. This conclusion would not, however, be justified if it could be shown that saponin is unable to penetrate the cell. The fact that in the highest concentrations employed[6] there was some

[6] Higher concentrations were not employed on account of the foaming which they produced.

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depression[7] of the rate of respiration indicates that saponin penetrates to some extent.

It is interesting to observe that the results obtained with *Bacillus subtilis* resemble those obtained by Osterhout in measuring the electrical conductivity of *Laminaria*, since in both cases the effect of saponin[8] is very much less than that of Na taurocholate.

SUMMARY.

1. The addition of Na taurocholate produces an increase in the rate of respiration at a concentration of 0.0000125 M, and a decrease at 0.001 M and in higher concentrations.

2. NaCl is antagonized by Na taurocholate, the most favorable proportion being 14,375 parts of NaCl to 1 part of Na taurocholate (molecular proportions).

3. Solutions of saponin, at concentrations from 0.00005 M to 0.001 M, decrease the rate of respiration: lower concentrations produce no effect.

[7] The depression was not due to the buffer action of the saponin. This was shown by comparing the change in pH produced in the saponin solution and in distilled water when a solution of carbonic acid was added to each.

[8] Unpublished results of experiments on *Laminaria*.

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COMPARATIVE STUDIES ON RESPIRATION.

XVI. Effects of Hypotonic and Hypertonic Solutions upon Respiration.

By O. L. INMAN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, January 15, 1921.)

Studies showing the effects of the osmotic pressure of the medium upon the organism have been numerous and various. Little work, however, has been done upon the relation of the production of carbon dioxide as affected by varying the osmotic pressure of the medium in which the organism lives.

Smith[1] reports that stems of *Tropæolum*, stems and roots of bean seedlings, and leaves of snowdrops, after deprivation of one-third to one-half of their total water by drying, respired more than normal plants. No increase in the respiration was observed on partly drying young stems of peony and asparagus.

Palladin and Sheloumova[2] observed that potato tubers, when allowed to lose water by drying in the air or by immersion in a 10 per cent solution of sodium chloride, in general showed a decrease in the amount of carbon dioxide produced.

Bailey and Gurjar[3] have investigated the respiration of seeds as related to the amount of water present. In general it is shown the respiration falls off as the moisture diminishes.

Warburg[4] stated that hypertonic solutions may increase the consumption of oxygen by fertilized eggs of the sea urchin as much as three or four times. Loeb and Wasteneys[5] repeated these experi-

[1] Smith, A. M., *Rep. Brit. Assn. Advancement Sc.*, 1915–16, lxxxv, 725.

[2] Palladin, V. I., and Sheloumova, A. M., *Bull. Acad. Sc. Petrograd*, 1918, 801, abstracted in *Chem. Abstr.*, 1918, xii, 1889.

[3] Bailey, C. H., and Gurjar, A. M., *J. Agric. Research*, 1918, xii, 685.

[4] Warburg, O., *Z. physiol. Chem.*, 1909, lx, 443.

[5] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 469.

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ments using a different species (*Strongylocentrotus purpuratus*) and found no increase when sufficient NaCl was added to increase the specific gravity of the sea water by about 50 per cent. They found an increase in the case of unfertilized eggs (as did Warburg) but they attributed this to the fact that the hypertonic solution causes the formation of a membrane. Unfertilized eggs which had already formed membranes (as the result of treatment with butyric acid) showed no increase in hypertonic solutions. Measurements by the writer of the specific gravity of the solution described by Loeb and Wasteneys showed it to be about 1.036, while that of the most concentrated solution used by the writer was much above this.

Using the method of measuring respiration described by Osterhout,[6] the writer undertook to measure the production of carbon dioxide while the osmotic pressure of the medium was changed sufficiently to be highly hypertonic on the one hand and quite hypotonic on the other. The marine alga *Laminaria agardhii* was used as the basis of most of the work. The results with *Ulva lactuca* were quite similar to those with *Laminaria*.

It was found that the carbonates of sea water interfered with the measurement of the carbon dioxide produced, and to overcome this difficulty the chamber containing the *Laminaria* was lined with filter paper dipped in artificial sea water (van't Hoff's solution, containing no carbonates). No other liquid was added but the chamber was so small that a very slight amount of evaporation sufficed to saturate the air. The change thereby produced in the concentration of the solution in the thallus was negligible and the cells may be regarded as bathed in a solution of constant concentration during the experiment. Uniform normal respiration was also obtained when the *Laminaria* was immersed in artificial sea water or placed in the respiration chamber without the addition of liquid or moistened filter paper.

The pieces of *Laminaria* were selected from fresh material as nearly uniform in size as possible and placed in the container. The normal rate of respiration was then obtained for each piece of tissue before it was treated. The pieces were then removed and exposed to the hypertonic or hypotonic solution for 5 minutes, at the end of

[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

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which time they were placed in the chamber (with a little of the same liquid as that with which they had been treated) and the rate of respiration was then measured at intervals of 10 to 20 minutes, until the expiration of 60 minutes.

The concentrated solutions of sea water were obtained by slow evaporation of normal sea water both with and without the use of heat.

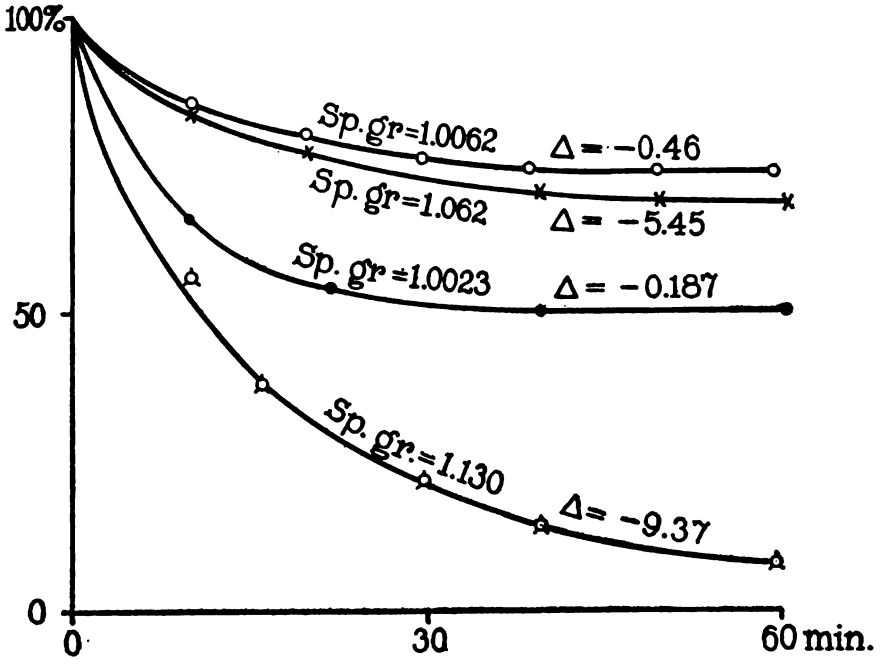

FIG. 1. Curves showing the rate of respiration of *Laminaria* during exposure to sea water of various specific gravities and freezing point depressions. Δ for Woods Hole sea water is normally − 1.81 and the specific gravity 1.024. The ordinates represent the rate of production of CO_2 expressed as per cent of the normal. The normal rate represents a change in pH from 7.78 to 7.36 in from 1.5 to 2 minutes. The abscissæ represent time in minutes. Average of 3 or more experiments: probable error of the mean less than 10 per cent of the mean.

No difference was found in the results they produced when of the same concentration. Artificial sea water (van't Hoff's solution, without the carbonates) in high concentration affected respiration in the same manner as ordinary concentrated sea water. The specific gravity of the sea water was measured by means of a Westphal balance and

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the depression of the freezing point was approximately determined by means of a Beckmann apparatus. The temperature of these experiments was 18° ± 2°C.

Fig. 1 shows quite clearly that in higher concentrations, *e.g.* specific gravity 1.130 ($\Delta = -9.37$) there was always a decrease in the amount of carbon dioxide given off. It is also shown that with hypotonic solutions the decrease in respiration is not so pronounced.

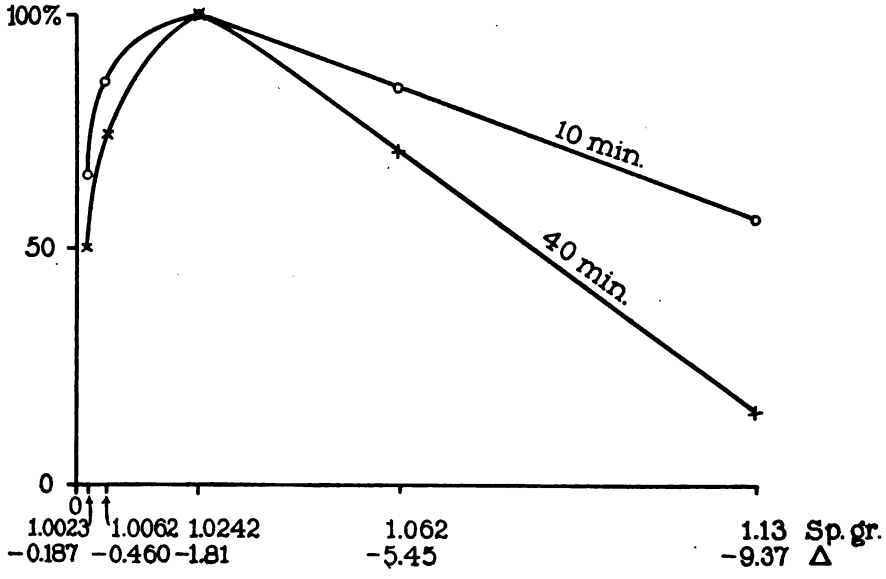

FIG. 2. Curves showing the rate of respiration of *Laminaria* in relation to the specific gravity and freezing point depression of sea water. The upper curve shows per cent of respiration at end of 10 minutes for all concentrations and dilutions. The lower curve shows the same at end of 40 minutes. The point at the intersection of the curves represents normal sea water. These curves are constructed from the same data as the curves in Fig. 1. The ordinates represent rate of production of CO_2 expressed as per cent of the normal. Abscissæ represent specific gravity; under each of the numbers denoting specific gravity is noted the corresponding freezing point depression.

Fig. 2 shows the relation between the specific gravity of the solution and the respiration to be almost a linear function in the case of hypertonic solutions. In the case of hypotonic solutions it is not so simple.

Experiments of a similar nature were carried out on wheat seedlings in which hypertonic solutions alone were used. Hypertonic solutions,

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of the same specific gravity as sea water (specific gravity 1.024), of sodium chloride and calcium chloride were tried; also a mixture of sodium chloride and calcium chloride in the molecular proportion of 50:1. The results were all similar to those produced by the use of hypertonic sea water on *Laminaria* in that there was always a fall in the rate of respiration.

SUMMARY.

1. In highly hypertonic solutions of sea water the rate of respiration of *Laminaria agardhii* is rapidly reduced.

2. In highly hypotonic solutions the rate of respiration of *Laminaria agardhii* is reduced somewhat less rapidly than in the case of hypertonic solutions.

3. Hypertonic solutions of NaCl, $CaCl_2$, and of mixtures of NaCl and $CaCl_2$ in the proportion of 50:1, all caused a decrease in the rate of respiration of wheat seedlings.

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FURTHER OBSERVATIONS ON THE PRODUCTION OF PARTHENOGENETIC FROGS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 19, 1921.)

The writer has repeatedly published[1] short reports on the production of frogs from unfertilized eggs by Bataillon's method of puncturing the membrane of the egg with a fine needle. The writer has succeeded in raising over twenty of these parthenogenetic frogs to an advanced and some to an adult stage. Two such specimens (leopard frogs) are represented in Fig. 1, together with a scale giving their size. They were at the time of death 13 and 14 months old respectively, and the death of these, as of the other specimens, was due to intestinal infection. The parthenogenetic frogs were apparently normal in every respect.

The second point of interest is the fact that both sexes occur among the parthenogenetic frogs. Three females were obtained among over twenty males, yet the preponderance of males may have been simply an accident. Fig. 2 gives a macroscopic photograph of ovaries and kidneys of one parthenogenetic female, and Fig. 3 a microphotograph of a section through the ovary. The fact that both sexes occur suggests that in the frog the female may be heterozygous for sex.

It was ascertained that the male parthenogenetic frogs and tadpoles possess a diploid and not a haploid number of chromosomes. The writer had the good fortune of obtaining the expert advice of Professor Richard Goldschmidt,[2] and later of Doctor Parmenter,[3] on this problem. Both authors found unquestionably a diploid number of chromosomes in the males. Parmenter was able to count definitely twenty-

[1] Loeb, J., and Bancroft, F. W., *J. Exp. Zool.*, 1913, xiv, 275; 1913, xv, 379. Loeb, J., *Proc. Nat. Acad. Sc.*, 1916, ii, 313; 1918, iv, 60; The organism as a whole, from a physicochemical viewpoint, New York, 1916.

[2] Goldschmidt, R., *Arch. Zellforsch.*, 1920, xv, 283.

[3] Parmenter, C. L., *J. Gen. Physiol.*, 1919–20, ii. 205.

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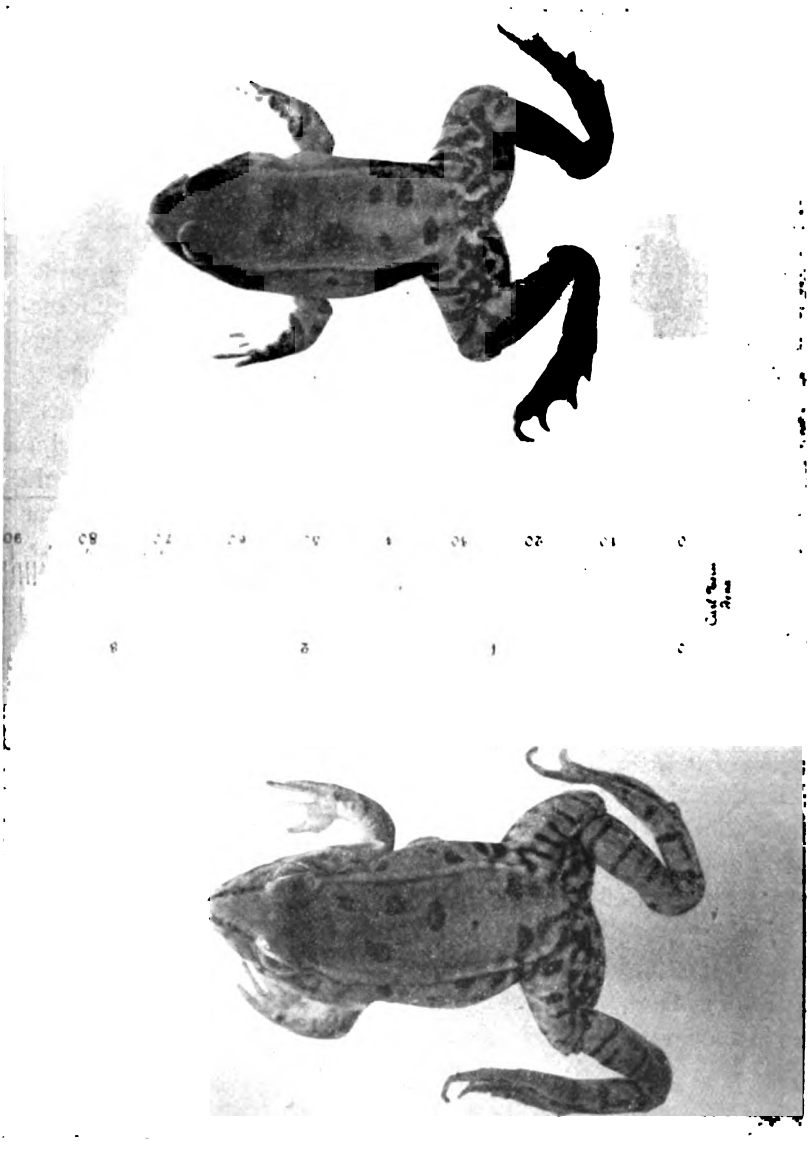

FIG. 1. Two adult male parthenogenetic leopard frogs, natural size (preserved in formaldehyde). The one to the left lived from Feb. 27, 1917, to Mar. 24, 1918; the one to the right from Mar. 16, 1916, till May 22, 1917

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six chromosomes in a number of specimens. The question arises how to account for the fact that the number of chromosomes is diploid. The first thought might be that the eggs had been accidentally fertilized, but this is excluded by the mode of procedure. As is well known, the eggs of the frog are fertilized outside the body of the

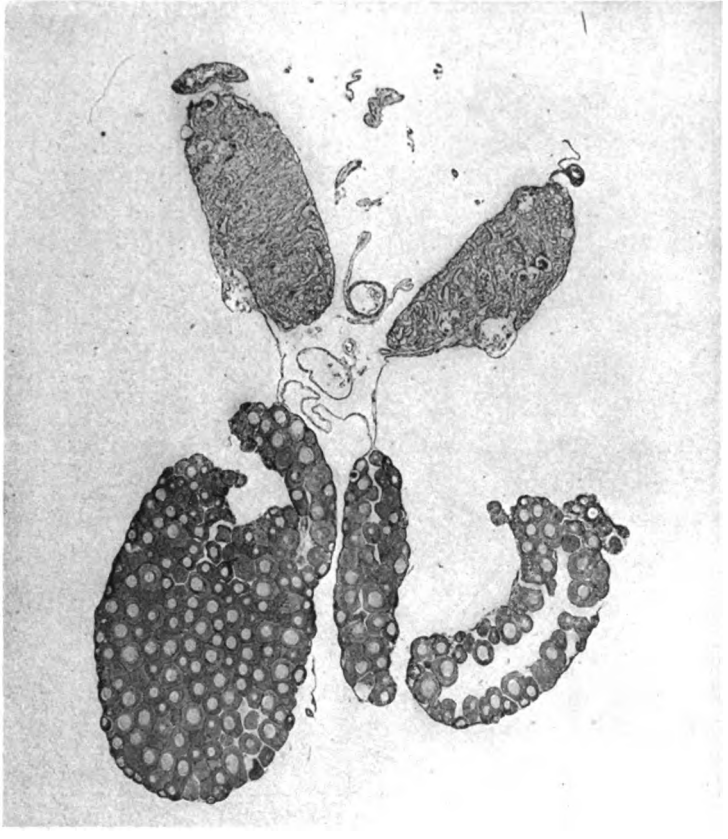

FIG. 2. Ovaries and kidneys of a parthenogenetic female frog.

female, and the females used for our experiments had not yet commenced to lay their eggs. The females after having been killed were submersed in 95 per cent alcohol and left there for several minutes to kill any sperm that might have stuck to the outside skin. The skin was cut open and the eggs were removed from the oviduct with

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sterilized instruments. The hands of the experimenter were also sterilized. About 50 to 100 eggs were put on each glass slide and the eggs of every second or third slide were not punctured, serving as controls. In no case did a single control egg show any development;

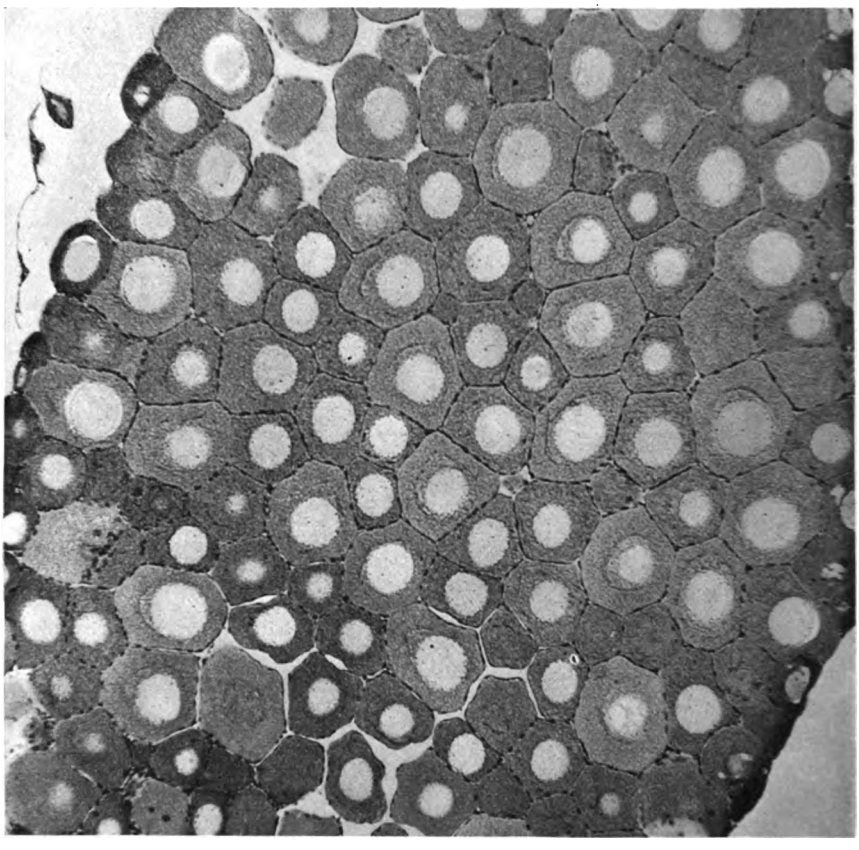

FIG. 3. Section through the ovary shown in Fig. 2.

only punctured eggs developed. This excludes the idea that the eggs were fertilized.

There remain other possibilities to account for the fact that the chromosome number is diploid. As Parmenter states[3]

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"The diploid number, as well as the similarity in form of the tetrads of the parthenogenetic and normal animals, may have been brought about by the retention of the second polar body, or by a premature division of the chromosomes without the division of the cell body just before the first cleavage."

Brachet[4] had previously counted the number of chromosomes in a parthenogenetic tadpole, 18 days old, and found a diploid number, but, of course, it was out of the question to ascertain the sex of the tadpole.

In my last publication on the subject I mentioned the possibility that the parthenogenetic females might have the haploid number of chromosomes. This question can only be decided by an actual count of the chromosomes in female parthenogenetic frogs. It is perhaps of interest in this connection that Hovasse[5] in an investigation of the number of chromosomes of young parthenogenetic tadpoles of frogs reports to have found both the diploid number and the haploid number. Since it is not possible to determine the sex of early tadpoles the observations of Hovasse do not answer the question whether or not the female parthenogenetic frog possesses a haploid number of chromosomes. There is, however, no doubt left that some if not all of the parthenogenetic male frogs possess a diploid number of chromosomes.

We usually received in one shipment a large number of female frogs either from South Carolina or from Chicago as soon as the spawning season began. It took about half a day to puncture the eggs of one female frog and as a consequence some time elapsed before the eggs of every female in the lot were punctured. It was found that tadpoles of good vitality were obtained only from the eggs of the first and second frogs used for the experiment. The eggs of the frogs which were punctured later, after the frogs had been in the laboratory for 2 days or more, either gave no tadpoles at all or if tadpoles were produced they died in less than 3 weeks, while the tadpoles from the eggs punctured immediately after the arrival of the frogs went on developing although the conditions under which the eggs were kept were the same in all cases (see Table I).

[4] Brachet, A., *Arch. Biol.*, 1911, xxvi, 362.

[5] Hovasse, R., *Compt. rend. Acad. Sc.*, 1920, clxx, 1211.

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TABLE I.

Date of experiment.	Female No.	Approximate No. of eggs punctured.	No. of tadpoles hatching.	No. of tadpoles that died within 3 weeks.
1919				
Mar. 25	I	8,500	92	21
" 26	II	5,000	43	22
" 28	III	3,000	9	9
" 29	IV	3,000	2	2
" 30	V	100	0	0
" 30	VI	400	0	0
" 30	VII	200	0	0
" 31	VIII	1,500	13	13
" 31	IX	150	0	0
" 31	X	1,300	1	1
" 31	XI	No eggs used.		
Apr. 1	XII	2,800	13	13
" 1	XIII	1,700	0	0
" 1	XIV	1,400	0	0
" 2	XV	1,200	16	14
" 2	XVI	200	0	0
" 2	XVII	1,100	1	1
" 3	XVIII	1,800	16	16
" 3	XIX	2,000	16	12
" 4	XX	1,500	0	0
" 4	XXI	None.		
" 4	XXII	1,700	0	0
" 5	XXIII	2,000	0	0

The results of a series of experiments made on the eggs of a second lot of frogs received later confirmed these results (Table II).

TABLE II.

Date of experiment.	Female No.	Approximate No. of eggs punctured.	No. of tadpoles hatched.	No. of tadpoles that died within 3 weeks.
1919				
Apr. 8	XXIV	2,000	6	5
" 8	XXV	4,000	14	14
" 9	XXVI	3,000	2	2
" 9	XXVII	3,500	0	0
" 10	XXVIII	2,000	0	0

It is, therefore, obvious that only those eggs were capable of developing which were punctured on the 1st or 2nd day while later they lost their power of developing or lost their vitality. It may be

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possible that the eggs must be punctured at a certain stage, *e.g.* at the period after the first polar body is given off and before the second polar body is extruded in order to develop or in order to develop normally, but this can only be decided by further experiments.

In these experiments the eggs were not covered with blood since it was found in previous experiments that this did not improve the yield of parthenogenetic tadpoles.

Many, sometimes the majority, of the parthenogenetic tadpoles did not metamorphose even within a year although they grew normally. It was suspected that this was due to an imperfect development of the thyroid gland or to lack of iodine in the food. In one tadpole, over a year old, the attempt was made to bring about metamorphosis by the feeding of thyroid gland from cattle. Three feedings were sufficient to cause metamorphosis within 2 weeks.

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CHEMICAL AND PHYSICAL BEHAVIOR OF CASEIN SOLUTIONS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 6, 1921.)

1. L. L. Van Slyke and J. C. Baker described in 1918[1] a method for preparing "pure casein" from skimmed milk, which consisted in "the gradual addition of acid and its immediate distribution through the mass of milk without causing coagulation of casein at the point where the acid first comes into contact with a portion of the milk. This result can be accomplished by introducing the acid below the surface of the milk with simultaneous high-speed mechanical stirring. . . . After standing under gentle stirring for 3 hours with acidity just below the point of casein coagulation, addition of acid is continued slowly, accompanied as before by rapid stirring in order to obtain the particles of casein coagulum in the finest possible state of division." The coagulated casein is then centrifuged and after repeated washings is found free from Ca and P. As Van Slyke and Baker point out, the pH of this casein coagulum is about 4.5 to 4.6; *i.e.*, it is slightly below the isoelectric point. The essential feature of Van Slyke and Baker's method, therefore, consists in slowly bringing the milk or casein solution approximately to the pH of the isoelectric point of casein. The writer has shown that gelatin gives off all ionogenic impurities at the isoelectric point[2] and Van Slyke and Baker's experiments show that the same method works also with casein. The casein prepared after Van Slyke and Baker's method is also free from albumin since this latter protein is soluble at pH 4.5 or 4.7, and is hence removed from the insoluble isoelectric casein by washing.

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

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In our experiments we used casein prepared after Van Slyke and Baker's method from skimmed milk and in addition from a commercial "pure casein." Both preparations gave practically the same result. In order to remove traces of fat from the casein the latter was washed in acetone.

2. In previous publications the writer had shown that weak dibasic and tribasic acids combine in molecular proportions with crystalline egg albumin, prepared after Sörensen and with gelatin.[3] It can be shown that the same is true for casein. 1 gm. of isoelectric casein, prepared after Van Slyke and Baker, was put into 100 cc. of watery solution containing 1, 2, 3, etc. cc. of 0.1 N HCl or 0.1 N H_3PO_4. The pH of the casein solution was ascertained potentiometrically and the number of cc. of 0.1 N acid required to bring the 1 per cent casein solution to the same pH was plotted as ordinates over the final pH of the casein solution as abscissæ. The casein chloride or casein phosphate is not completely soluble in a 1 per cent solution at room temperature until the pH is about 3.0 or a trifle below. When too much acid is added, *i.e.* when the pH is 1.6 or possibly a little above, casein precipitates out again from a 1 per cent solution.

Fig. 1 gives the curves for HCl and H_3PO_4, drawn out within those limits of pH within which the casein salts are soluble in a 1 per cent solution at room temperature. The curves show that about three times as many cc. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of originally isoelectric casein in a 1 per cent solution to the same pH; or in other words, H_3PO_4 combines with casein in molecular proportions, as we should expect if casein phosphate is a true chemical compound.

It was not possible to plot the corresponding curves for casein sulfate and casein oxalate since these salts are too sparingly soluble. This is true also for casein salts with other acids; *e.g.*, triacetic acid.

3. The writer had shown that the influence of different acids on the physical properties of gelatin or crystalline egg albumin depends only upon the valency and not upon the nature of the ion in combination with the protein.[3] Thus the values of osmotic pressure or viscosity of gelatin chloride are identical with those of gelatin phosphate for

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559; 1920–21, iii, 85, 247; *Science*, 1920, lii, 449.

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the same pH and the same concentration of originally isoelectric gelatin; and the same is true for crystalline egg albumin. The reason is that in the case of gelatin or albumin phosphate the anion is the

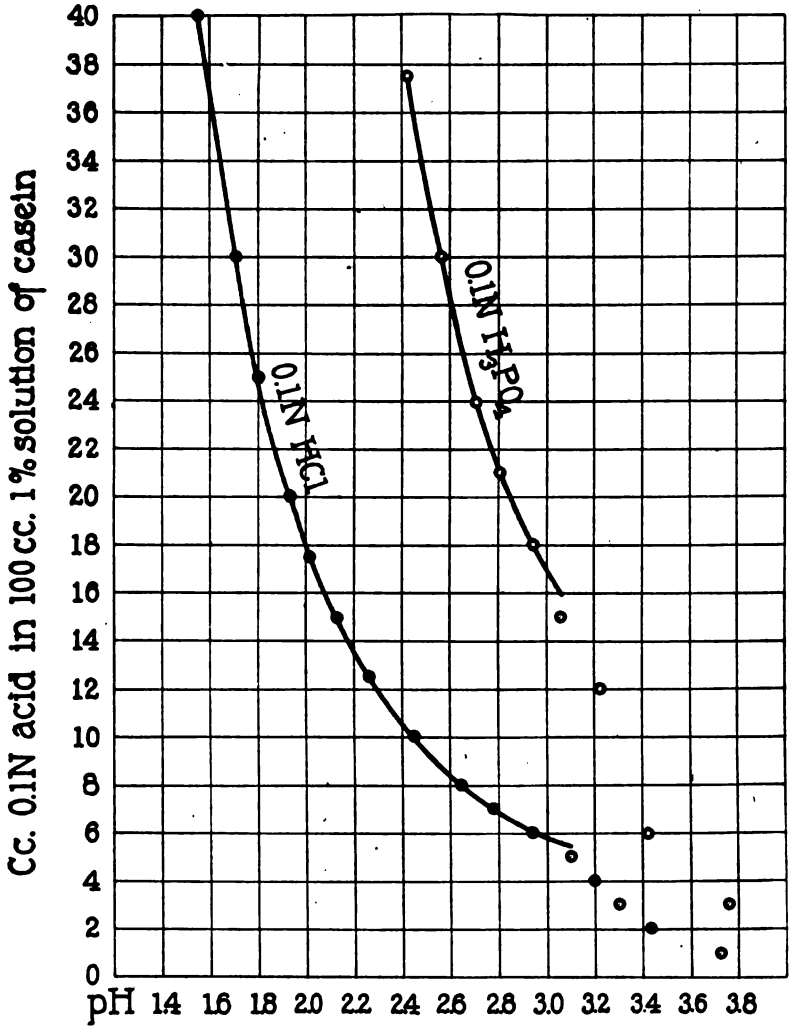

FIG. 1. Ordinates represent the cc. of 0.1 N HCl or H_3PO_4 in 100 cc. of 1 per cent casein solution. The abscissæ are the pH of the solution. Approximately three times as many cc. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of casein to the same pH.

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monovalent anion H_2PO_4 and not the trivalent anion PO_4. If the same rule holds for casein, the osmotic pressure and viscosity of casein phosphate should be practically identical with that of casein chloride when plotted over the same pH and when the concentration of (originally isoelectric) casein is the same in both cases (1 gm. in 100 cc. of solution). Fig. 2 shows that the osmotic pressure curves for casein chloride and casein phosphate (in 1 per cent solutions) are

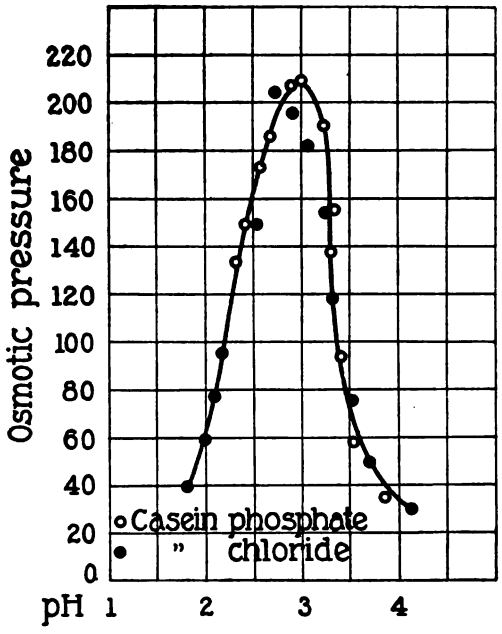

FIG. 2. Osmotic pressure of 1 per cent solutions of casein chloride and casein phosphate as function of pH. The two curves are almost identical.

almost identical. The curve includes also the osmotic pressure at a pH between 4.0 and 3.0 where the two casein salts are not completely soluble in 1 per cent solutions, but since the relative solubilities of casein chloride and casein phosphate are also practically identical, the osmotic pressure curves for the pH where the solubility of the two salts is not complete remain approximately the same.

Fig. 3 gives the viscosity curve for 1 per cent solutions of casein chloride and casein phosphate over pH as abscissæ. The ordinates

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are the quotients of the time of outflow of the casein solutions over the time of outflow of distilled water through the same viscometer. The time of outflow for distilled water was 60 seconds. We will, for the sake of brevity, call this ratio specific viscosity. We see that this specific viscosity does not rise above 1 as long as the pH is above

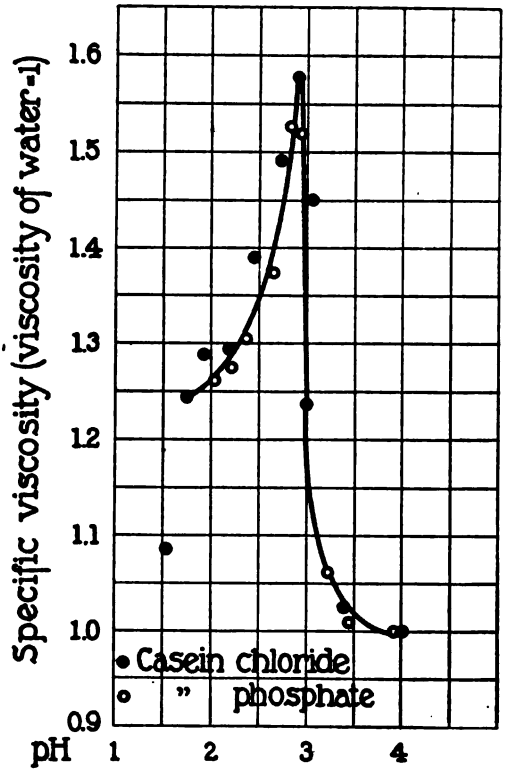

FIG. 3. Viscosity of 1 per cent solutions of casein chloride and casein phosphate. The curves are approximately identical.

3.5. At a pH of 3.0 a sharp rise occurs, because the solubility of the casein increases at this point considerably. With a further fall of pH the viscosity diminishes again. Fig. 3 shows that the viscosity curves for casein chloride and casein phosphate are almost identical, which was to be expected if the rules found for gelatin and crystalline egg albumin are also true for casein.

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We had shown that when we add $Ca(OH)_2$ or $Ba(OH)_2$ to isoelectric gelatin or isoelectric crystalline egg albumin the two alkalies combine with the protein in equivalent proportion.[3] Hence the same number

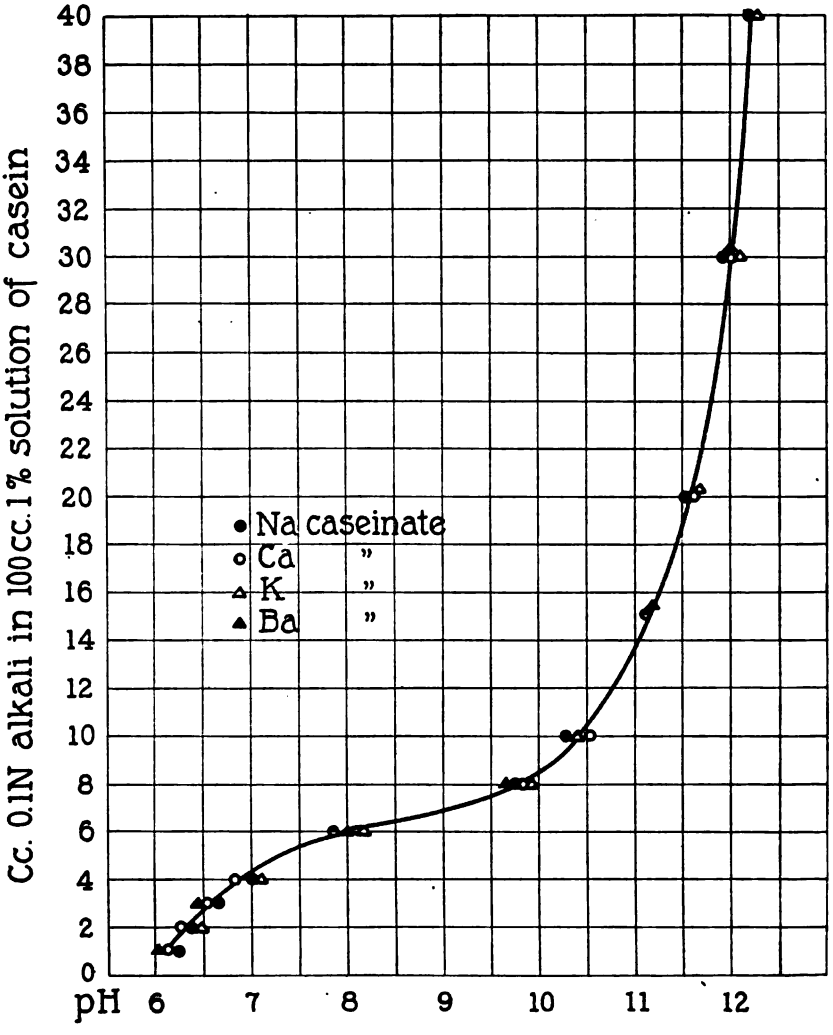

FIG. 4. Ordinates are the cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ in 100 cc. of 1 per cent solution of casein. Abscissæ are the pH of the solution. The curves for the four alkalies are identical, proving that Ba and Ca combine with casein in equivalent proportion.

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of cc. of 0.1 N $Ba(OH)_2$ or $Ca(OH)_2$ was required to bring a 1 per cent solution of isoelectric gelatin or crystalline egg albumin to a given pH as was required in the case of NaOH or KOH. It can be shown

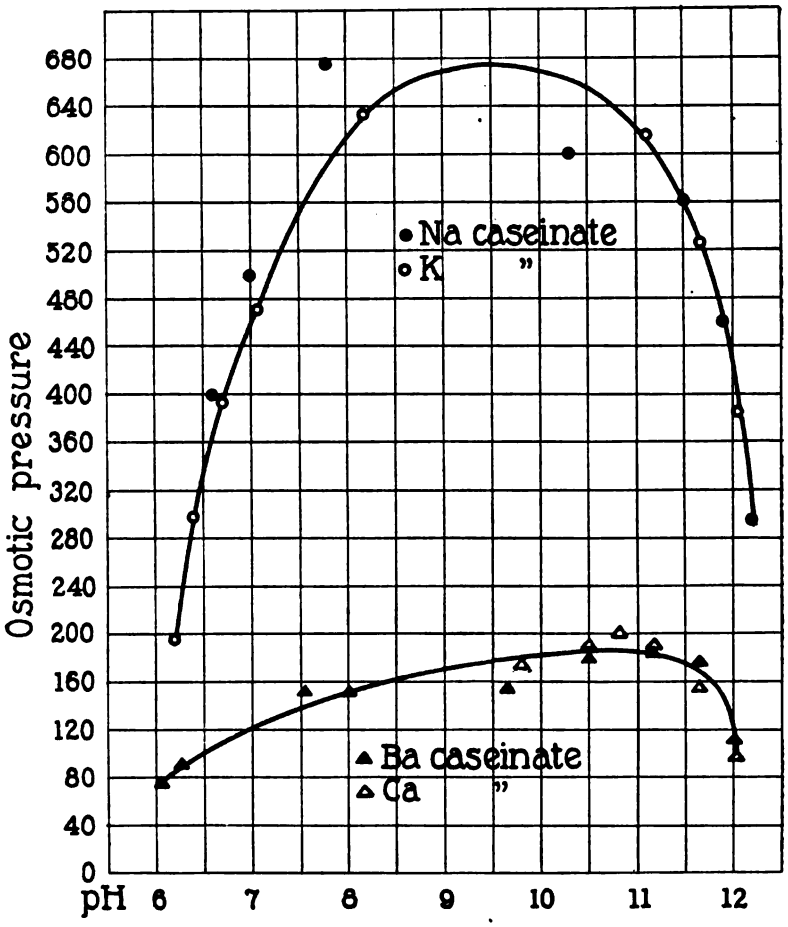

FIG. 5. Osmotic pressure of Na caseinate, K caseinate, Ba caseinate, and Ca caseinate. The curves for the former two caseinates are identical and considerably higher than those for the latter two caseinates.

that the same is true for casein. In Fig. 4 the abscissæ are the pH, the ordinates the number of cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ that must be contained in 100 cc. of a 1 per cent solution

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of isoelectric casein to bring it to the same pH. The curves for the four alkalies are identical (Fig. 4), thus proving that $Ca(OH)_2$ and $Ba(OH)_2$ combine with casein in equivalent proportion. Table I gives the limits of pH between which the four metal caseinates are completely soluble in a 1 per cent solution at room temperature.

TABLE I.

1	per	cent	Na	caseinate	completely	soluble	between	pH	7.02	and	> 12.20
1	"	"	K	"	"	"	"	"	7.09	"	> 12.28
1	"	"	Ca	"	"	"	"	"	10.53	"	> 12.00
1	"	"	Ba	"	"	"	"	"	10.50	"	> 12.26

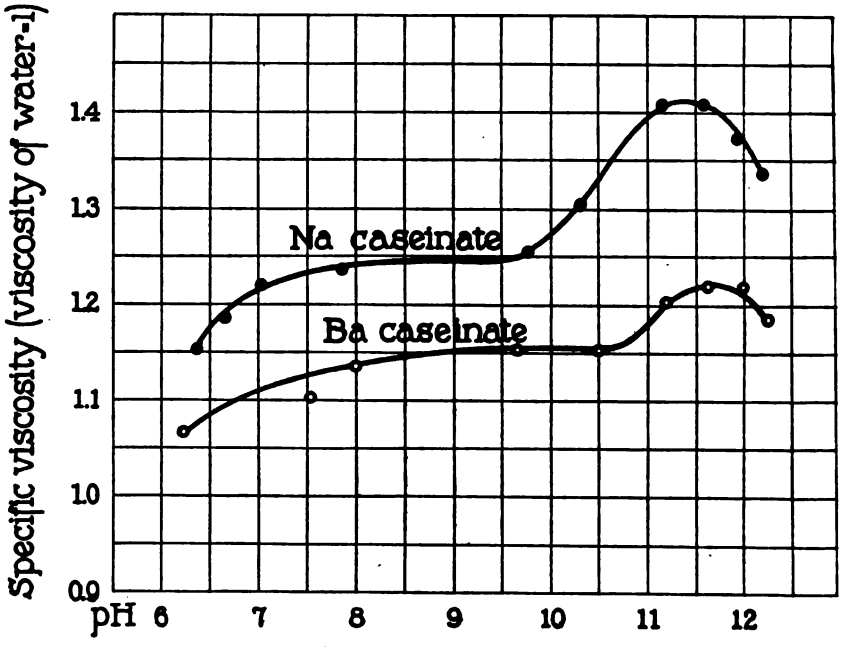

FIG. 6. Viscosity curves for Na caseinate and Ba caseinate.

On account of the incomplete solubility of Ba caseinate and Ca caseinate at a pH between 4.7 and 10.5 it is not possible to draw any conclusion from the relative osmotic pressure or relative viscosity of metal caseinates with monovalent and divalent cation between a pH of 4.7 and 10.5. This should be kept in mind in judging the curves in Figs. 5 and 6.

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When we plot the osmotic pressure of these solutions as ordinates over the pH as abscissæ (Fig. 5), we notice that the curves for the osmotic pressure of Na and K caseinate are alike; the curves for the casein salts are, however, over three times as high when the cation is monovalent (Na or K) than when the cation is bivalent (Ca or Ba). This is, however, chiefly the result of the fact that Ca and Ba caseinate are incompletely soluble up to a pH of 10.5.

Fig. 6 gives the viscosity curves for Na caseinate and Ba caseinate. The difference in height between the two casein salts is between pH 11 and 12 of a similar order as in the case of Na and Ba gelatinate.

SUMMARY AND CONCLUSION.

The experiments on casein solutions therefore confirm the conclusion at which we arrived from the behavior of gelatin and crystalline egg albumin that the forces determining the combination between proteins and acids or alkalies are the same forces of primary valency which also determine the reaction between acids and alkalies with crystalloids, and that the valency and not the nature of the ion in combination with a protein determines the effect on the physical properties of the protein.

The measurements mentioned in this paper were made by Dr. E. Brakeley, Mr. M. Kunitz, and Mr. N. Wuest of this Laboratory, to whom I wish to express my indebtedness.

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THE COLLOIDAL BEHAVIOR OF PROTEINS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 31, 1921.)

I.

Colloids show a number of peculiarities which at first appearance seem to be lacking in crystalloids, and these properties are generally accounted for by differences in the degree of dispersion. We have repeatedly discussed these peculiarities in our analysis of the chemical behavior of proteins, *e.g.* the depressing effect of neutral salts on the osmotic pressure, swelling, and viscosity of certain proteins; the peculiar influence of the hydrogen ion concentration of the solution on these properties; and finally the peculiar influence of the valency of the ion with which the protein is in combination and the apparent lack of influence of the other chemical properties of the ion except valency and sign of charge.[1] The dispersion theory accounts for these difficulties by the assumption of differences in the degree of aggregation of the protein particles. If, for example, the addition of some salt to a protein solution of a definite pH lowers its osmotic pressure, the assumption is made that the salt diminishes the degree of dispersion of the colloidal particles in solution. It is not possible to submit the dispersion theory to a quantitative test since we cannot measure the degree of dispersion of a protein.

A second theory to account for the influence of salts and hydrogen ion concentration is Pauli's ionization theory which ascribes the osmotic pressure, the swelling, and viscosity chiefly to the hydratation of ionized protein, while the non-ionized protein molecule is assumed not to be hydrated. The idea of such a hydratation of protein ions has become doubtful in view of recent experimental and theoretical investigations by Lorenz[2] and by Born,[3] and, moreover, the writer

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391; *Science*, 1920, lii, 449.

[2] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.

[3] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

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has been able to show that the conductivity measurements of protein solutions contradict Pauli's ionization theory.

The solution of the problem seems to lie in a field altogether foreign to the speculations current in colloid chemistry, namely in the Donnan equilibrium, which exists when a membrane separates two solutions, one of an electrolyte for the ions of which the membrane is permeable, and one of an electrolyte for one ion of which the membrane is not permeable.[4] It is immaterial whether the latter ion is a colloid or a crystalloid; it is only necessary that it cannot diffuse through the membrane. When a collodion membrane separates a gelatin or albumin chloride solution of pH 3.3 from an aqueous solution of HCl of originally the same pH (but without gelatin), the pH is no longer the same on both sides of the membrane at the time of equilibrium but is lower on the outside than in the gelatin solution. The Donnan equilibrium demands in this case that acid be given off from the gelatin chloride solution to the outside aqueous solution (containing no gelatin). The writer has found that, *e.g.* a 1 per cent gelatin chloride solution of pH 3.5 is in equilibrium with an aqueous HCl solution of about pH 3.0.[5]

II.

Gelatin chloride solutions containing 1 gm. of originally isoelectric gelatin in 100 cc. solution and having a pH of 3.5 were made up in H_2O and in different concentrations of $NaNO_3$ varying from M/4,096 to M/32 $NaNO_3$, all of pH of 3.5. These solutions were put into collodion bags connected with a manometer to measure the final osmotic pressure of the solution. The collodion bags were put into HCl solution of pH 3.0 made up in water and different concentrations of $NaNO_3$, the pH of the $NaNO_3$ solutions being also 3.0. These outside solutions contained no gelatin. The collodion bags were put into these aqueous solutions free from gelatin in such a way that the concentration of the $NaNO_3$ solution inside the collodion bag was always the

[4] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

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same as outside. The osmotic pressure of the 1 per cent gelatin chloride solution, which was after 18 hours 435 mm. when no salt was present, was only 63 mm. when the inside and outside solutions were made up in M/32 $NaNO_3$.

Table I gives the influence of the concentration of $NaNO_3$ on the osmotic pressure. It is obvious that the osmotic pressure diminishes with the concentration of the salt. This phenomenon had already been described by Lillie[6] and by the writer.[7] Donnan has shown that this depressing effect of a salt on the osmotic pressure of a colloidal solution is a necessary consequence of his theory of membrane equilibrium, and this conclusion is supported by the following experiments.

TABLE I.

Original inside solution, 1 per cent gelatin chloride of pH 3.5 made up in various concentrations of $NaNO_3$ of the same pH.

Outside solution, HCl of pH 3.0 made up in the same concentrations of $NaNO_3$ of pH 3.0 as the inside solution.

	Concentration of $NaNO_3$.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
Osmotic pressure in mm. of H_2O..	435	405	371	335	280	215	134	85	63
P.D. inside solution in millivolts.....	+31	+26	+24	+22	+16	+12	+7	+4	0

The writer undertook measurements of the potential difference between the gelatin chloride solutions inside the collodion bag and the aqueous solutions outside the collodion bag with the aid of a Compton electrometer. It was found that the gelatin chloride solution was always positively charged while the outside aqueous solution was negatively charged, as was to be expected. The second important fact is that the P.D. diminishes with the increase in concentration of the neutral salt added and (if the necessary corrections are made) in approximately the same ratio as the osmotic pressure diminishes (lower row in Table I).

[6] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 391.

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III.

What is the origin of these potential differences? Beutner[8] has shown that the potential differences at the boundary of water and water-immiscible substances obey Nernst's well-known logarithmic formula

$$E = \frac{RT}{nF} \ln \frac{C_1}{C_2}$$

or at room temperature and for $n = 1$

$$0.058 \log \frac{C_1}{C_2}$$

Loeb and Beutner have found the same formula to hold for the potential differences at the boundary of water and the skin of an apple, a tomato, or the leaf of a rubber plant.

It can be shown that the potential differences mentioned in Table I follow Nernst's formula, if we assume that only the hydrogen ion concentration need be considered for the potential difference. If C_1 is the concentration of free HCl in the gelatin solution, and C_2 the concentration of HCl in the outside aqueous solution (without gelatin) the value $\log \frac{C_1}{C_2}$ becomes equal to (pH inside − pH outside).

We measured the pH of the gelatin chloride solution (inside solution) and of the outside HCl solution (without gelatin) after the osmotic and the Donnan equilibria were established. The surprising result was noticed that the difference of pH inside the gelatin solution minus the pH of the outside solution (without gelatin) becomes the smaller the greater the concentration of the $NaNO_3$, as shown in Table II.

We can calculate from this difference of pH inside minus pH outside the P.D. between inside and outside solution in millivolts by multiplying the differences by 58 or 59 (correcting for temperature of 24°C.). If the Nernst formula holds the values for P.D. thus calculated should be identical with the observed values for P.D. Table III shows that this is true to a remarkable degree.

[8] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

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TABLE II.

pH Inside Minus pH Outside after 18 Hours.

Original inside solution, 1 per cent originally isoelectric gelatin dissolved in various concentrations of $NaNO_3$ made up with HCl to pH 3.5.

Outside solution, same concentrations of $NaNO_3$ all made up with HCl to pH 3.0.

	Concentration of $NaNO_3$.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
pH of inside solution.....	3.58	3.56	3.51	3.46	3.41	3.36	3.32	3.29	3.25
" " outside "	3.05	3.08	3.10	3.11	3.14	3.17	3.20	3.22	3.24
Difference pH inside minus pH outside.....	0.53	0.48	0.41	0.35	0.27	0.19	0.12	0.07	0.01

TABLE III.

Potential Difference between Gelatin Solution and Outside Solution.

Concentration of $NaNO_3$.	Calculated by Nernst's formula from pH.	Observed.
	millivolts	*millivolts*
0	31.2	31
M/4,096	28.3	28
M/2,048	24.0	24
M/1,024	20.7	22
M/512	16.0	16
M/256	11.2	12
M/128	7.0	7
M/64	4.1	4
M/32	0	0

IV.

The greatest puzzle in the physical chemistry of the proteins is the fact that at the isoelectric point of gelatin and crystalline egg albumin the osmotic pressure is a minimum, that it rises when acid is added, at first with the increase of acid added, reaching a maximum when the pH is about 3.5, and that on further addition of acid a rapid fall of osmotic pressure occurs.

It was also reported in the preceding papers that the osmotic pressure of gelatin chloride and gelatin phosphate solutions of the same pH and the same concentration of originally isoelectric gelatin was about the same, that the osmotic pressure of gelatin oxalate was slightly

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lower, while that of gelatin sulfate was only half as high or not quite half as high as that of gelatin chloride.

Measurements of the potential differences between the gelatin solution and the outside solution revealed the fact that the curves presenting the P.D. as a function of the hydrogen ion concentration resemble the curves for osmotic pressure. The P.D. curves have a minimum at the isoelectric point, rise steeply with increasing hydrogen ion concentration until a pH of 3.9 is reached, then drop equally steeply again when the pH falls further. Moreover, the maximum of the P.D. curve for gelatin sulfate is about one-half of that of the maximum of the P.D. curve for gelatin chloride and the P.D. curve for gelatin chloride is about equal to the P.D. curve for gelatin phosphate, while that of gelatin oxalate is only slightly lower than that of gelatin chloride.

The next question was whether or not the Nernst formula can account for these differences. The pH of the gelatin solutions and of the outside solutions were measured at the point of equilibrium and it was found that the difference of pH inside minus pH outside multiplied by 58 gave approximately the number of millivolts actually measured. The agreement between the calculated P.D. and the observed P.D. was not as perfect as in the case of the salt effect (Table III) but sufficient to leave no doubt that Nernst's theory accounts for these P.D.

V.

Procter[9] has applied the Donnan equilibrium to the theory of swelling of gelatin, reaching the conclusion that swelling is an osmotic phenomenon and that the amount of swelling of a gelatin chloride solution is determined by the concentration of the free ions inside the gel minus the concentration of the free ions in the outside solution. He did not measure the pH. By filling this gap the writer was able to satisfy himself that the depressing influence of salts upon the swelling of gelatin is due to a diminution of the difference of pH inside

[9] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

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and outside the gel, and that the curves expressing the influence of neutral salts on the value of pH inside minus pH outside the gel, and on the swelling run approximately parallel.

SUMMARY AND CONCLUSION.

1. It is well known that neutral salts depress the osmotic pressure, swelling, and viscosity of protein-acid salts. Measurements of the P.D. between gelatin chloride solutions contained in a collodion bag and an outside aqueous solution show that the salt depresses the P.D. in the same proportion as it depresses the osmotic pressure of the gelatin chloride solution.

2. Measurements of the hydrogen ion concentration inside the gelatin chloride solution and in the outside aqueous solution show that the difference in pH of the two solutions allows us to calculate the P.D. quantitatively on the basis of the Nernst formula $E = \frac{RT}{nF} ln \frac{C_1}{C_2}$ if we assume that the P.D. is due to a difference in the hydrogen ion concentration on the two sides of the membrane.

3. This difference in pH inside minus pH outside solution seems to be the consequence of the Donnan membrane equilibrium, which only supposes that one of the ions in solution cannot diffuse through the membrane. It is immaterial for this equilibrium whether the non-diffusible ion is a crystalloid or a colloid.

4. When acid is added to isoelectric gelatin the osmotic pressure rises at first with increasing hydrogen ion concentration, reaches a maximum at pH 3.5, and then falls again with further fall of the pH. It is shown that the P.D. of the gelatin chloride solution shows the same variation with the pH (except that it reaches its maximum at pH of about 3.9) and that the P.D. can be calculated from the difference of pH inside minus pH outside on the basis of Nernst's formula.

5. It was found in preceding papers that the osmotic pressure of gelatin sulfate solutions is only about one-half of that of gelatin chloride or gelatin phosphate solutions of the same pH and the same concentration of originally isoelectric gelatin; and that the osmotic pressure of gelatin oxalate solutions is almost but not quite the same as that of the gelatin chloride solutions of the same pH and concentra-

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tion of originally isoelectric gelatin. It was found that the curves for the values for P.D. of these four gelatin salts are parallel to the curves of their osmotic pressure and that the values for pH inside minus pH outside multiplied by 58 give approximately the millivolts of these P.D.

In this preliminary note only the influence of the concentration of the hydrogen ions on the P.D. has been taken into consideration. In the fuller paper, which is to follow, the possible influence of the concentration of the anions on this quantity will have to be discussed.

The writer wishes to express his indebtedness to his technical assistants, Mr. M. Kunitz and Mr. N. Wuest, who have carried out the measurements in these experiments.

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METHODS OF STUDYING THE RESPIRATORY EXCHANGE IN SMALL AQUATIC ORGANISMS, WITH PARTICULAR REFERENCE TO THE USE OF FLAGELLATES AS AN INDICATOR FOR OXYGEN CONSUMPTION.

By H. MUNRO FOX.

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(Received for publication, March 5, 1921.)

The results described below are a sequel to those already published in this *Journal* on the spontaneous aggregation of flagellates.[1] In that publication it was shown that a flagellate, *Bodo sulcatus*, forms aggregations in regions where the concentration of dissolved oxygen is an optimum for it. This optimum is less than the saturation concentration of oxygen dissolved in water under atmospheric partial pressure. The flagellates move out of regions where the oxygen concentration is above or below the optimum to gather into the optimal regions. They are positively chemotactic to a certain concentration of dissolved oxygen.

This behavior of the flagellates can be made use of to indicate changes in the concentration of dissolved oxygen due to the respiration of an aquatic organism present in the water. For if the organism under investigation be kept motionless in a suspension of the flagellates in water saturated with oxygen at the atmospheric partial pressure, the flagellates will collect into those regions where the oxygen concentration is lowered through the respiratory activity of the organism. The sizes of the aggregations of flagellates thus formed will show the relative amounts of oxygen absorbed by the different parts of the surface of the organism.

The following experiment will show how the method is applied. A small fresh water invertebrate, such as a *Chironomus* larva, is

[1] Fox, H. M., An investigation into the cause of the spontaneous aggregation of flagellates and into the reactions of flagellates to dissolved oxygen, *J. Gen. Physiol.*, 1920–21, iii, 483, 501.

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placed on a slide in a few drops of liquid from a *Bodo* culture. A cover-glass, supported at its four corners by wax feet, is placed over the liquid and is pressed down so that the larva is just prevented from moving, without being injured by the pressure (Fig. 1). The flagellates in the neighborhood of the larva swim in to its surface,

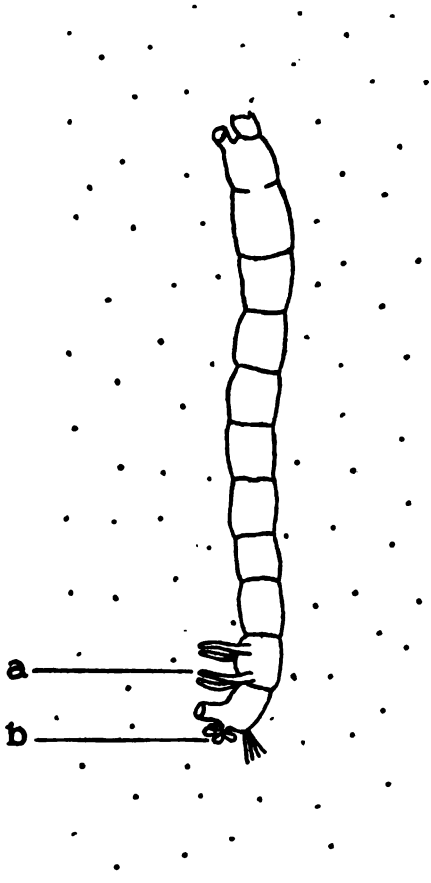

FIG. 1.

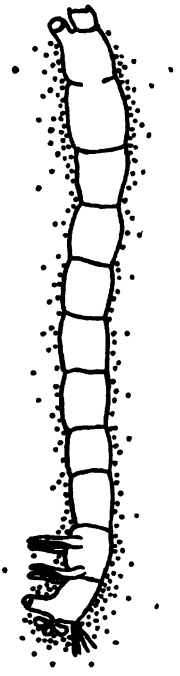

FIG. 2.

FIG. 1. *Chironomus* larva which has just been placed in a suspension of flagellates. *a*, ventral gills; *b*, anal gills. In all diagrams the density of the dots represents the density of distribution of the flagellates.

FIG. 2. The same larva shortly afterwards. The flagellates collect on the surfaces of the larva which are absorbing oxygen.

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collecting there in ever increasing numbers (Fig. 2). This collection is not a surface energy phenomenon which might be caused by any solid object in the suspension, for if a larva which has just been killed by being dropped into hot water is used in place of the living larva, there is no aggregation of flagellates at its surface. The *Bodo* are simply attracted towards the surface of the living larva because in this neighborhood the respiratory activity of the insect has lowered the concentration of oxygen dissolved in the water. The reason for the collection is the same as that for the spontaneous aggregation previously described.[1] The flagellates move into a region of lower oxygen concentration caused, in the case of spontaneous aggregation, by their own respiration, in this experiment by the respiration of the larva. Here, of course, the aggregation of flagellates takes place much more rapidly than in spontaneous aggregation for the larva consumes relatively much more oxygen than the flagellates. If spontaneous aggregation occurred rapidly, it would interfere with the collection of the flagellates on the respiratory surfaces of the larva. The danger of spontaneous aggregation is, however, averted by a preliminary filtering of the *Bodo* suspension through bolting-silk. This not only removes fragments of debris from the culture jar but the exposure of the water to the air during filtration allows it to become saturated with oxygen. This greatly retards subsequent spontaneous aggregation.[1]

More flagellates collect on some parts of the insect's surface than on others. These are the most actively respiring surfaces. When the aggregation has reached certain dimensions, a clear space free from flagellates appears between it and the surface of the larva (Fig. 3). This is due to the same cause as the appearance of an area free from flagellates in the center of a spontaneous aggregation; the dissolved oxygen has been reduced to a certain low concentration at which the flagellates leave the region. The aggregation of flagellates on the surface of the larva now gradually becomes a band separated from the surface by a clear space, this happening first at those surfaces which are most actively respiring. Eventually the flagellates move out everywhere from the surface of the larva and come all to lie in a band encircling it (Fig. 4). In the case of an animal, such as a *Simulium* pupa, which respires only in one part, namely through

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the walls of certain filamentous appendages, no oxygen at all being absorbed by the rest of the body surface, the aggregation and band of flagellates have the forms shown in Fig. 5. In all cases the band gradually enlarges, moving out to a stationary equilibrium position just within and parallel to the edges of the cover-glass. This is the same phenomenon as the final stage of spontaneous aggregation and band formation.

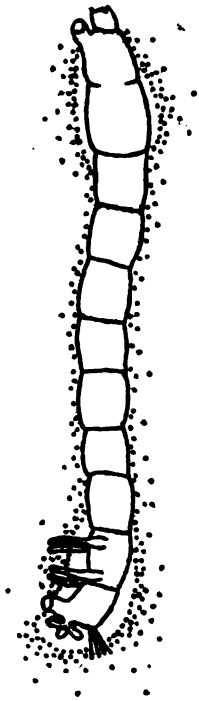

FIG. 3.

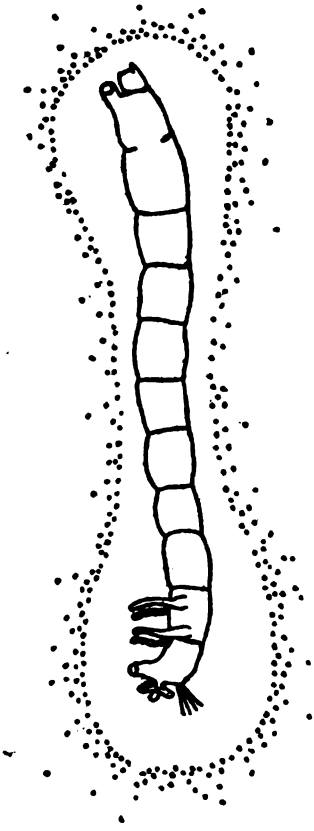

FIG. 4.

FIG. 3. The same larva a little later. The flagellates leave those surfaces of the larva which by their more active respiration have first reduced the concentration of dissolved oxygen below the optimum for the flagellates.

FIG. 4. The same larva. The flagellates have all left the surface of the larva and lie in a gradually spreading band in the zone of optimum oxygen concentration.

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By this method the relative amounts of oxygen absorbed by different surfaces of a small aquatic organism can be estimated very delicately. Most oxygen is taken in at those places where most flagellates accumulate and where, later on, the aggregation first moves away from the surface in the form of a band. The use of the flagellates as an indicator in such work has the more value in that they can be cultivated either in fresh or in salt water. Throughout the present investigation the flagellate employed was *Bodo sulcatus*. It was obtained by steeping grass from the garden behind the laboratory at Plymouth in tap water.

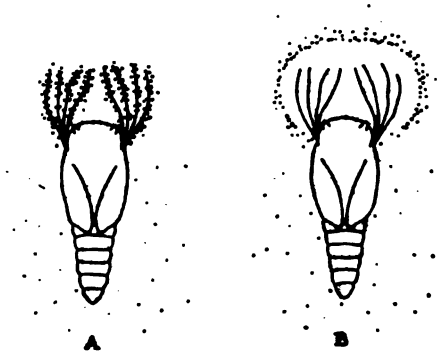

FIG. 5. *Simulium* pupa in a suspension of flagellates. A, the flagellates collect on the surfaces which are absorbing oxygen; B, later, the flagellates leave the region where the oxygen concentration has fallen below the optimum for them.

The use of flagellates for detecting the intake of oxygen by the surfaces of organisms is complementary to Engelmann's well known method of demonstrating the output of oxygen in photosynthesis by means of bacteria.[2]

The flagellates have been used so far to test the respiratory activities of several types of aquatic insect larvæ, but the work so far has been done mostly from the point of view of perfecting the methods. In the future it is proposed to examine systematically all available types of larvæ with the particular object of settling the functions of the several kinds of so-called gills. To make the meaning clearer, the work done with the red *Chironomus* larva will now be outlined.

[2] Engelmann, T. W., Neue Methode zur Untersuchung der Sauerstoffausscheidung pflanzlicher und thierischer Organismen, *Arch. ges. Physiol.*, 1881, XXV, 285.

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These larvæ have on the ventral surface of the eighth abdominal segment four tube-like hollow outgrowths, in which the blood circulates. On the dorsal surface of the last abdominal segment there are four shorter hollow outgrowths, also with a blood stream through them. All these processes are thin-walled and have a thin cuticle. It is usually assumed that the chief respiratory gas-exchange takes place through the walls of these outgrowths, the so-called ventral and anal gills. When the larvæ are tested with the *Bodo* suspension, it is found that oxygen is absorbed by the whole of the general body surface of the larva except the head which has a very thick cuticle. The intensity of absorption is usually greatest in the posterior abdominal segments, but this is not invariably so. The most actively respiring segments vary from one individual to another, and when a single individual is tested a number of successive times the most actively respiring regions may vary in position each time. However, the remarkable result is the behavior of the so-called gills. No more oxygen is found to be absorbed by the "anal gills" than by the general body-surface and no oxygen at all is absorbed by the "ventral gills." The latter may be seen to project through and beyond the collection or band of flagellates without influencing it (Figs. 2 and 3).

This result can be confirmed by quite another mode of experimentation. The blood of these larvæ contains hemoglobin dissolved in it. When a larva is examined with a microspectroscope the whole body shows the oxyhemoglobin absorption bands. If a larva is now placed in water in a hollow-ground slide and covered with a cover-slip, the edges of which are sealed down with vaseline, at the end of about 20 minutes the whole body of the larva shows the reduced hemoglobin absorption spectrum. The larva does not die when this has occurred. It will remain alive with heart beating for many hours beneath the sealed cover-slip. If a small bubble of air is now allowed to enter beneath the cover-slip, so that it comes to rest up against the surface of the larva, an examination with the microspectroscope shows that oxyhemoglobin first appears inside the body of the larva at a point nearest to the bubble and thence it spreads over the rest of the body. Thus the part of the body-surface nearest to the bubble first absorbs the oxygen, and not the "gills." But a more striking result than this can be obtained. If by chance the bubble

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comes to rest in contact with the "ventral gills," the first appearance of oxyhemoglobin is inside the abdominal segment nearest to the bubble and not within the "gills" themselves. This fully bears out the result obtained with the flagellates. Whatever be the function of the "ventral gills," it is not that of absorbing oxygen.

The spectroscopic method is one suited to this particular larva alone; it cannot be applied where hemoglobin is absent. The flagellate method, however, is one of general application.

At the same time that the oxygen intake was studied, the relative output of carbon dioxide by different surfaces of the body was investigated. This was done by mounting the larva on a slide in the same manner as for studying the oxygen absorption, but, in place of a suspension of flagellates, an indicator was used which changes color as carbon dioxide goes into solution, altering the hydrogen ion concentration of the water. The position and extent of the color change indicates the place and amount of the carbon dioxide output. Several indicators are available which change in color about the neutral point of water. Neutral red and rosolic acid were tried but were found unsuitable for these experiments because in the thin layer of solution between cover-slip and slide the colors are not sufficiently intense to show a sharp change. The indicator which was found suitable for use was a solution of hematoxylin with just sufficient alkali added to make it a bluish pink. In regions where carbon dioxide is being given off by the larva into the water the bluish pink color changes through orange to yellow. The color change is sharp even in the thin layer of liquid between cover-slip and slide. It can be more exactly observed when the examination is made under a very low power of the microscope, such as a 2 inch objective. All tests were discarded in which the larva defecated or in which any liquid at all came out through the anus, for the rectal fluid is alkaline.

Using the red *Chironomus* larva it was found by this method: (1) that carbon dioxide is given off by the whole body surface except by the head and the ventral gills; (2) that most carbon dioxide is given off by the posterior abdominal segments, but that the relative amounts given off by different segments vary in different individuals and in the same individual at different times; (3) that carbon dioxide is not always given off by the different surfaces of an

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individual larva in the same relative amounts that oxygen is absorbed by these surfaces; (4) that the "anal gills" give off no more carbon dioxide than the general body surface; and (5) that the ventral gills give off no carbon dioxide.

It is intended to apply the flagellate method of studying the oxygen intake and the indicator method of studying the carbon dioxide output to small aquatic types of the various groups of the animal kingdom, but first of all a systematic examination will be made of aquatic insect larvæ with special reference to the functions of the so-called blood gills and tracheal gills and to the relation of closed tracheal systems to respiration.

SUMMARY.

1. Flagellates are positively chemotactic to a certain concentration of dissolved oxygen which is lower than that in water saturated with oxygen under atmospheric partial pressure. Consequently, when a small aquatic animal is held motionless between cover-slip and slide in a suspension of flagellates in water saturated with oxygen, the flagellates are attracted to those parts of the animal which are absorbing oxygen. The relative sizes of the flagellate aggregations then show the relative activities of the different surfaces of the animal in absorbing oxygen.

2. Applying this method to the red *Chironomus* larva it was found that the animal respires by the whole body surface except by the head and the "ventral gills" and that the relative intensity of oxygen intake by the different parts of the body varies in different individuals and in the same individual at different times.

3. The absence of oxygen intake by the "ventral gills" was confirmed with the microspectroscope. In oxygen-free water all the hemoglobin of the blood becomes reduced. When an air bubble is now introduced so that it touches the "ventral gills" oxyhemoglobin first appears in the nearest body segment to the bubble, not in the "gills."

4. When a small aquatic animal is held motionless between cover-slip and slide in a solution of an indicator which changes color about the neutral point of water the relative extent of color change at different surfaces of the animal's body indicates the relative amounts of carbon dioxide given off by these surfaces.

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5. Using this method with the red *Chironomus* larva similar conclusions were reached for carbon dioxide output as for oxygen intake.

The work was done in August and September, 1919, at the Laboratory of the Marine Biological Association, Plymouth. I wish to thank the Director and staff for their constant kindness and assistance. I wish also to thank the trustees of the Ray Lankester Fund for having nominated me to an Investigatorship and Sir Ray Lankester, K.C.B., F.R.S. for having lent me a microspectroscope.

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THE PHAGOCYTOSIS OF SOLID PARTICLES.

III. Carbon and Quartz.

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(From the Laboratory of Applied Physiology, Harvard Medical School, Boston.)

(Received for publication, January 15, 1921.)

It has been shown by Haldane (1) and by Mavrogordato (2) that silicious dust when inhaled tends to remain in the lungs, causing phthisis. Coal dust, on the other hand, tends to move out of the lungs and is, therefore, harmless. The different behavior of silicious and carbonaceous dusts in the lungs is also the cause of the abnormally high mortality from tuberculosis among silicious miners and the abnormally low mortality among coal miners (3). Without going into a discussion of the mechanism of dust removal from the lungs, it is sufficient to state that the first step appears to be always the ingestion of the dust particles by phagocytic cells in the alveoli. It seemed probable, therefore, that a study of the phagocytosis of carbonaceous and silicious particles would show that the former are ingested more readily than the latter, in agreement with the clinical facts, and might throw some light on the cause of this difference. This was found to be true.

It became at once evident, in undertaking a comparison between the rates of phagocytosis of carbon and quartz particles, that the usual method of incubating them together with the leucocytes in a common suspension might yield nothing more than a comparison of the relative chances of collision of the two kinds of particles with the leucocytes. It has, in fact, been shown in two preceding papers on the phagocytosis of quartz (4) and carbon (5) particles that the more rapid ingestion of large particles by leucocytes compared to small ones can be accounted for quantitatively by the fact that a large particle moves faster when stirred up in a suspension with leucocytes, and therefore comes into collision with more cells in a given time. In this paper the same methods will be applied to a

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study of the comparative rates of phagocytosis of carbon and quartz particles to determine whether or not carbon is ingested by leucocytes more readily than would be predicted from the calculated chances of collision.

The method of calculating R, the chance of collision, and the experimental procedure are described in the earlier papers (4, 5). It must

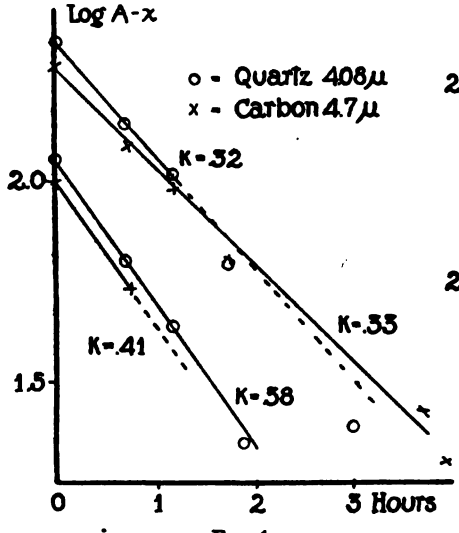

FIG. 1.

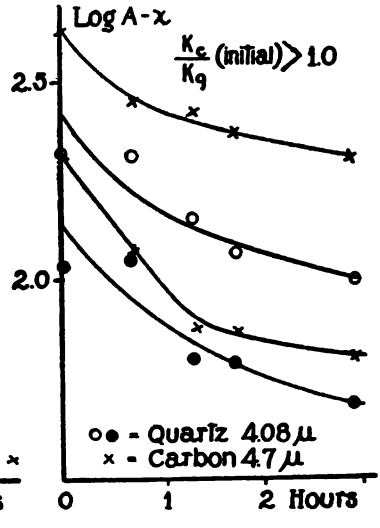

FIG. 2.

FIG. 1. Ordinates represent logarithms of the number of particles outside the leucocytes in 0.02 c. mm. of the suspension. Curves show that the actual rates of ingestion of the quartz and carbon were nearly equal. Quartz, being, heavier, collides more often with the cells. The corrected ratio, $\frac{K \text{ carbon}}{K \text{ quartz}} = \frac{1.06}{0.36} = 3$, shows that carbon is taken up 3 times as readily as quartz. Two concentrations of both quartz and carbon were used, the higher concentration being twice the lower. See Table II.

FIG. 2. Ordinates and abscissae as in Fig. 1. Curves showing the rates of ingestion of quartz and carbon particles. If the experimental points for quartz are accurate the initial $K = 0$. Assuming that the first two points on the quartz curves are erroneous, as seems most probable, the initial ratio, $\frac{K \text{ carbon}}{K \text{ quartz}}$, is seen to be at least > 1. Even interpreting the curves thus in favor of the quartz, the corrected ratio is 2.8, showing that the carbon is taken up at least 2.8 times as readily. Two concentrations (in the proportion of 1 to 2) of both quartz and carbon were used.

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suffice here to state that R is proportional to the difference between the velocities of leucocytes and particles, $Vp - Vc$, and to the square of the sum of the diameters of cells and particles, $(C + P)^2$. The latter factor allows for the fact that a larger particle makes a larger target. The rate of phagocytosis is measured by K of a monomolecular reaction and is equal to the slope of the straight line obtained by plotting against time the logarithms of the *number of particles not yet ingested*, as determined by frequent counts on an ordinary hemocytometer. The cell suspensions were obtained from peritoneal exudates in rats. The phagocytic mixtures were rotated slowly on a revolving drum during incubation to prevent settling out of the cells or particles.

TABLE I.

Calculated Chances of Collision, R.

Nature of Particle.	Diameter.	R.
	μ	
Carbon	3.2	144
	4.7	248
Quartz	2.4	130
	4.08	697
	4.63	1299

The results of one such experiment are plotted in Fig. 1. Since ordinates represent the logarithms of the number of particles counted at time, t (abscissæ), *outside* the leucocytes, the steeper the slope of the curve the more rapid the phagocytosis. A straight line in this figure, *i.e.*, a constant K, indicates that the same percentage of the number of collisions occurring between cells and particles is resulting in ingestion throughout the experiment. As has previously been pointed out, it is only the *initial slope*, in cases where K is *not* constant, which is expected to agree with the theoretical predictions. In Fig. 1, the initial K's of the two carbon experiments are 0.33 and 0.41, and of the quartz, 0.38 and 0.32 the averages being 0.37 and 0.35 respectively; *i.e.*, carbon is taken up $\frac{0.37}{0.35}$ or 1.06 times as fast as the quartz. Reference to Table I, however, in which are tabulated the chances of collision of the three quartz and two carbon suspensions used in

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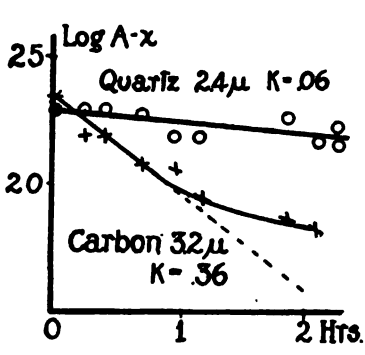

FIG. 3.

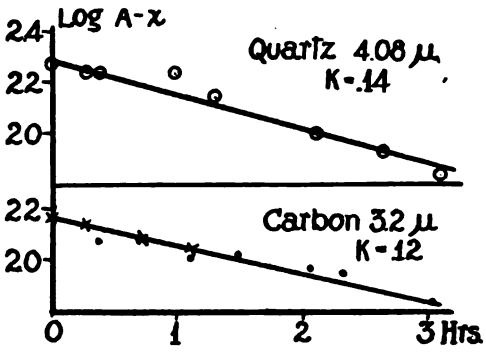

FIG. 4.

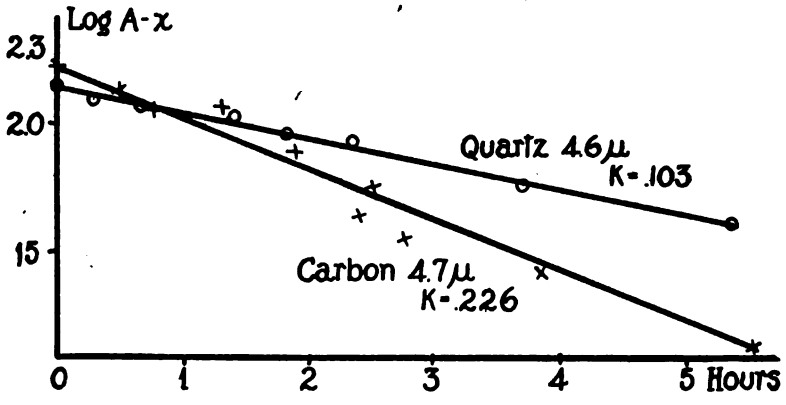

FIG. 5.

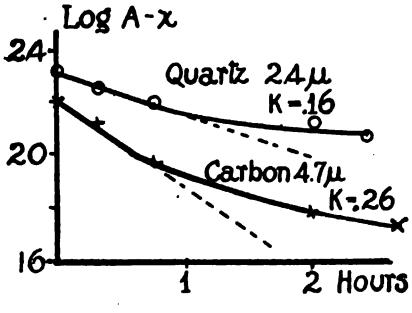

FIG. 6.

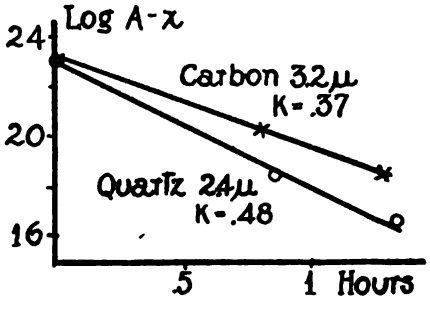

FIG. 7.

FIG. 3–7. Curves showing comparisons of rates of ingestion of quartz and carbon particles of various sizes by the suspension method. The logarithms of the number of particles in 0.02 c. mm. not yet ingested are plotted as ordinates against time in hours as abscissae. See Table II for the results of the comparisons when corrected for the chances of collision.

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these experiments, shows that these carbon particles (4.7μ) should collide with the cells only $\frac{248}{697}$ or 0.36 times as often as the quartz (4.08μ). It may be concluded, therefore, that carbon is ingested $\frac{1.06}{0.36}$ or 3 times as readily as quartz.

Results of all the other available comparisons between quartz and carbon are plotted in Figs. 2 to 8 and tabulated in Table II. In each experiment the initial K only was used in comparison unless K was constant throughout. The last column in Table II shows the number of collisions necessary for ingestion of one quartz particle if every collision with a carbon particle results in ingestion; *i.e.*, the

TABLE II.

Comparison of Rates of Phagocytosis of Carbon and Quartz by "Suspension" Method.

Fig.	Diameter of particles.		Initial K.		Ratio* $\frac{K \text{ carbon}}{K \text{ quartz}}$.		
	Carbon.	Quartz.	Carbon.	Quartz.	Observed.	Calculated.	Corrected.
1	4.7	4.08	0.37	0.35	1.07	0.36	3.0
2	4.7	4.08	0.03	0.03−	1.0+	0.36	2.8+
3	3.2	2.4	0.36	0.06	6.0	1.1	5.4
4	3.2	4.08	0.12	0.14	0.87	0.21	4.1
5	4.7	4.6	0.226	0.103	2.2	0.19	11.5
6	4.7	2.4	0.26	0.16	1.6	1.9	0.8
7	3.2	2.4	0.37	0.48	0.77	1.1	0.7

Average = 4.0

* The calculated ratios are the ratios of the chances of collision for the sizes of particles used as given in Table I. The corrected ratio is the quotient obtained by dividing the observed ratio by the calculated ratio.

value of the ratio $\frac{K \text{ carbon}}{K \text{ quartz}}$. In five out of seven experiments carbon is taken up 2.8 to 11.5 times as readily as quartz. In two instances only, due to some uncontrolled factor, quartz is taken up slightly more readily than carbon. The average of all shows that carbon is taken up 4 times as readily as quartz.

It should be observed that it is assumed that a large particle is taken up as easily as a small particle if the number of collisions are equal. That this is true, at least within limits of error of these experiments, was shown in the two previous papers (4, 5). It would appear

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from these figures that there is great variation in different experiments in the relative rates of ingestion of carbon and quartz, the ratio varying from 0.7 to 11.5. That this ratio may vary with the condition of the cells, even in the same experiment, will be shown later by another method.

Another contributing explanation is perhaps the agglutination of the carbon. In some experiments the carbon has been observed to be more stable than in others. This factor could always be controlled, however, by observation of the samples which were removed from the incubation mixtures at intervals for counting. Whenever any considerable agglutination was observed the experiment was discarded. With quartz, however, there was seldom any agglutination, and this is the most characteristic difference between the behavior of the two kinds of particles. Thus quartz can be thrown down repeatedly by centrifugalization, and when resuspended in water the particles are perfectly discrete. This cannot be done with carbon unless it is stabilized with acacia. This suggests that the cause of the more rapid agglutination may also be the cause of the greater speed of phagocytosis. One might suppose that if carbon agglutinates with carbon readily, it will also agglutinate readily with cells, whereupon ingestion promptly occurs.

Comparison of Quartz and Carbon by the "Film" Method.

Before proceeding to a discussion of this hypothesis, however, some experiments will be reported in which the rates of ingestion of carbon and quartz were compared by another method, which will be referred to as the "film" method. By this method, unlike the "suspension" method described previously, phagocytosis was allowed to proceed in a thin film between the slide and cover slip. Both cells and particles immediately settled out. The leucocytes crept about on the slide and ingested the particles.

A cover-slip was supported at its center and four corners by fragments of another fairly thick cover-slip. While held down with a small weight it was sealed at the corners with collodion. The weight prevented the cover-slip from floating up on the collodion, and made the distance from slide to cover uniform throughout. A thick suspension of cells with equal numbers of carbon and quartz particles

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was then allowed to run under the cover-slip and the edges were sealed with paraffin to prevent evaporation. The slide was at once placed on a warm stage at 37°C. and frequent counts were made of the number of free particles of quartz and carbon over a given area. The areas were measured by a disc micrometer in an ocular ruled in squares. It was customary to count sixteen such areas for each point.

The advantages of this method are:

1. The chance of collision depends merely upon the size which the particle offers as a target for the cells; i.e., $(C + P)$ which is so small a factor that it is almost negligible.[1]

2. Agglutination of particles is impossible.

3. Both quartz and carbon particles can be incubated together in the same film so that the number of active cells is identical at any one time.

The only disadvantage of the method is that the cells are injured by contact with the glass slide, so that the time curves do not follow the law for a monomolecular reaction as they otherwise would. Thus the cells are observed during the course of the reaction to spread out on the glass, becoming vacuolated and transparent and ceasing their activities.

Phagocytosis with the film method presents an interesting picture, as shown in the photographs (Figs. 8 and 9). Nearly all the cells are in active motion. The activities of one cell were recorded for 3 minutes during which time it refused one quartz particle and one carbon particle, and ingested three carbon particles, nearly reaching a fourth. No evidence was obtained that cells can sense particles from a distance, as reported by Commandon (6) for leucocytes ingesting starch grains, and by Schaeffer (7) for amebæ in proximity to carbon and glass. All the meetings seem to occur purely by chance. Sudden movements of leucocytes for a distance 2 or 3 times their own length have frequently attracted attention, as if the cell had been under

[1] C = diameter of cell, P = diameter of particle. This is merely the target factor from the formula for the chances of collision as used in the suspension method. In this instance, however, the chance of collision is $(C + P)$, not $(C + P)^2$, because motion is confined to two dimensions.

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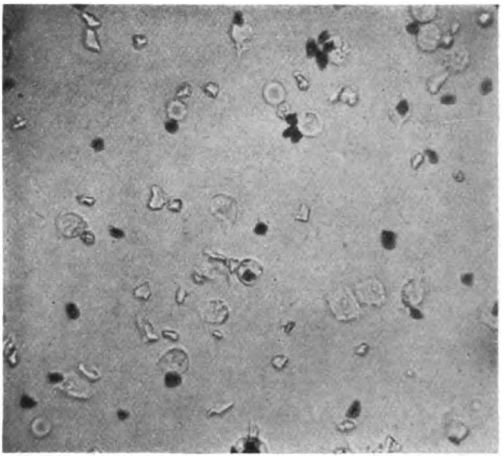

FIG. 8.

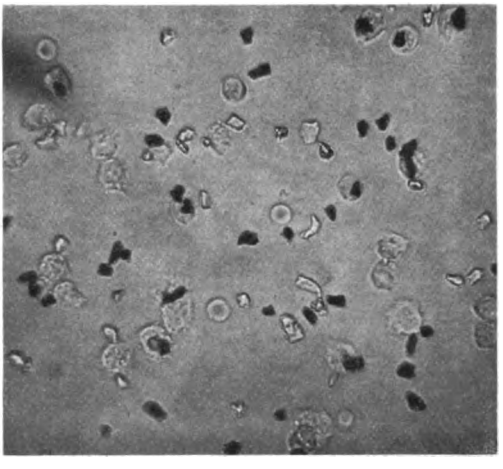

FIG. 9.

FIGS. 8 and 9. Photographs of quantitative comparison of the rates of phagocytosis of quartz and carbon particles by leucocytes of rats. The leucocytes are actively ameboid and many contain carbon particles. None of the available quartz particles, however, has yet been ingested. Attention is directed to the uniformity in the sizes of the quartz and carbon particles.

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tension by some long contracting pseudopod[2] which had suddenly succeeded in pulling the cell loose from its contact with the slide. Toward the end of an experiment the cells are usually aggregated in groups, mostly heavily laden with particles. This clumping is also found, apparently to the same extent, in a control preparation from which particles are omitted. Often cells are so full of particles that close inspection is necessary to see protoplasm at all.

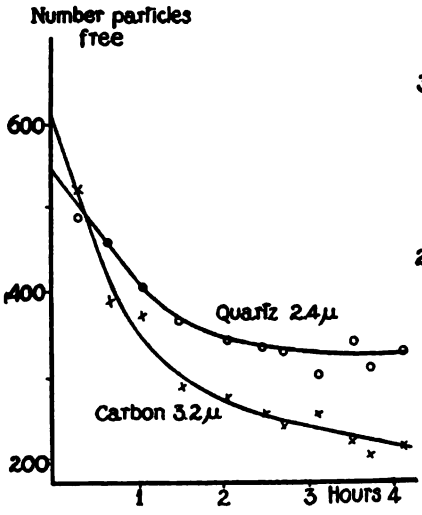

FIG. 10.

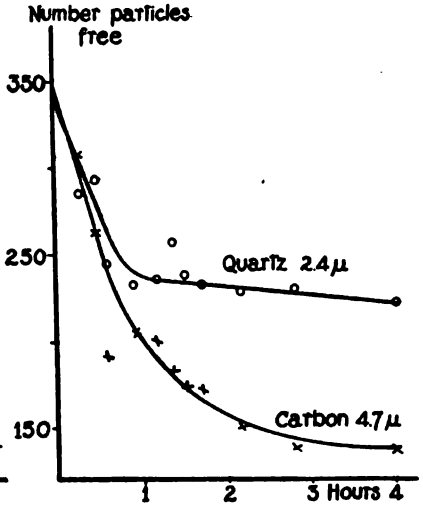

FIG. 11.

FIGS. 10 and 11. Comparison of phagocytosis of carbon and quartz by the film method. The numbers of particles not yet taken up in a given area are plotted as ordinates against time in hours as abscissae. Here no appreciable allowance need be made for the chances of collision, and carbon is obviously ingested more rapidly than quartz. See Table III for further analysis.

The results of two typical experiments of this sort are plotted in Figs. 10 and 11. Ordinates represent the number of particles of quartz or carbon counted over a given area which are still outside the cells. Time is plotted as abscissae. Inspection shows that the carbon is taken up more readily than the quartz. The question is how much more readily. Here the data can be analyzed with more

[2] Such pseudopods have been described by Kite (Kite, G. L., *J. Infect. Dis.*, 1914, xv, 319).

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assurance than with the suspension method. From Figs. 10 and 11 the number of particles ingested during each hour of the experiment has been determined graphically. This value divided by the average number of particles present outside the cells during that hour gives the percentage of particles taken up during the hour. These figures are recorded in Table III.

TABLE III.

Comparison of Rates of Ingestion of Quartz and Carbon Particles by "Film" Method.

Hrs.	Particles ingested per hr.		Experimental ratio* = $\frac{\text{per cent } C}{\text{per cent } Q}$	
	Carbon.	Quartz.		
	per cent	*per cent*		
0–1	54	29	1.9	Experiment 8.
1–2	22	19	1.1	Carbon 3.2 microns
2–3	15	4.5	3.3	Quartz 2.4 microns
3–4	13	1.6	8.1	Theoretical ratio 1.07
0–1	53	35	1.5	Experiment 9.
1–2	27	4.3	6.3	Carbon 4.7 microns
2–3	12	2.6	4.6	Quartz 2.4 microns
3–4	2.2	2.2	1.0	Theoretical ratio 1.20
0–1	57	27	2.2	Experiment 10.
1–2	27	5.9	4.6	Carbon 4.7 microns
2–3	3.5	1.2	2.9	Quartz 4.6 microns
				Theoretical ratio 1.01
0–1	44	15	2.9	Experiment 11.
1–2	22	11	2.0	Carbon 2.7 microns
2–3	10.5	4.9	2.1	Quartz 2.6 microns
				Theoretical ratio 1.01

* The experimental ratios are the ratios of the numbers of particles of carbon and quartz ingested per hour expressed in per cent of the average number present during that hour and $= \frac{\text{per cent Carbon}}{\text{per cent Quartz}}$. Data were obtained graphically from Figs. 10 and 11 for Experiments 8 and 9 and from similar unpublished figures for Experiments 10 and 11. The theoretical ratios are $\frac{R \text{ carbon}}{R \text{ quartz}}$. $R = C + P$ or the chance of collision, where $C = 9\mu$, the diameter of the leucocytes, and $P =$ the diameter of the particle.

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The ratio of these values $\left(\frac{\text{per cent Carbon}}{\text{per cent Quartz}}\right)$ for carbon and quartz (last column) is then a measure of the greater speed of ingestion of carbon. Strictly these figures should now be corrected by dividing by the ratio $\frac{(C + P) \text{ carbon}^1}{(C + P) \text{ quartz}}$ which is a measure of the relative chances of collision. Since the value of this factor is small (1.07 and 1.2), this correction has not been applied, but the necessary factor for correction is given for each comparison.

It is a very significant fact that this $\frac{\text{per cent Carbon}}{\text{per cent Quartz}}$ ratio increases after the 1st hour. This means that in comparison to carbon it is relatively harder to ingest quartz toward the end of the experiment than at the beginning. Thus, in Experiment 8 (Fig. 10), if every meeting between a cell and a carbon particle results in ingestion throughout the experiment, we may conclude that at the beginning of the experiment two meetings with quartz are necessary for ingestion, and at the end of the experiment, eight meetings. Eventually, carbon also is refused and the ratio $\frac{\text{per cent Carbon}}{\text{per cent Quartz}}$ necessarily decreases again. This happens in Experiment 9 (Fig. 11). If the phagocytic activity of the cells remained the same throughout an experiment, the percentage of carbon particles ingested during each hour would be constant. The rate of decrease of this percentage may, therefore, be taken as a measure of the rate at which the activity of the cells decreases. Thus, in Experiment 8, the phagocytic activity of the cells after 3 hours is only $\frac{13}{54}$ or 24 per cent of the original. This may be due to contact with the glass, agglutination of cells, decreased capacity of cells, or other factors.

Data from Experiments 10 and 11 are also included in Table IV. The former is the only exception to the rule that the ratio $\frac{\text{per cent Carbon}}{\text{per cent Quartz}}$ increases after the 1st hour. In this experiment both quartz and carbon (particularly quartz) were taken up more slowly even during the 1st hour than in the other three experiments, and it therefore seems probable that the decrease in the ratio in

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Experiment 11 may correspond to the decrease *following* the increase in Experiments 9 and 10.

The comparative rates of ingestion of carbon and quartz particles depend, therefore, upon the condition of the cells. Sometimes preparations of carbon and quartz have been made in which there was practically no ingestion of quartz. This may be seen in the photographs, Figs. 8 and 9, where no quartz is seen inside the cells though there is plenty available.

As far as the writer is aware the experiments in this paper constitute the first quantitative comparison of the effects of different kinds of solid substances on living cells. Even qualitative comparisons are limited. Aside from the effects of carbonaceous and silicious dusts in the lungs the only recorded observations seem to be the mere statement of Commandon (6) that starch is ingested by leucocytes more readily than carbon. Schaeffer (7) endeavored to compare the ingestion of carbon and glass particles by amebae but neither was ingested.

Phagocytosis of Carbon and Quartz by Sponge Cells

This experiment was done at the Marine Biological Laboratory, Woods Hole, with a small marine sponge, *Grantia.* The sponge was squeezed into a test-tube, and a thick suspension in sea water of active cells was obtained. 8 parts of this suspension were mixed with 4 parts of sea water concentrated to twice its normal strength by boiling, 1 part of 0.2 M borate mixture (pH 7.5), 1 part 10 per cent acacia neutralized with sodium hydroxide, and 2 parts of a suspension of 3.2 micron carbon particles and 2.4 micron quartz particles in distilled water. The result is a suspension of cells, quartz, and carbon in normal sea water plus 0.6 per cent acacia to stabilize the carbon and quartz, the alkalinity being approximately the same as that of normal sea water.

A sample of this mixture was allowed to run under a cover-slip supported as for the film method and sealed with paraffin. The cells were slowly ameboid and ingested the particles rather sluggishly compared to leucocytes. After 5 hours at room temperature, counts were made of the number of quartz and carbon particles found inside

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the cells. Only solitary cells were included in the count as it would be easy to overlook the colorless quartz particles in the aggregated cells. All the solitary cells in each field were examined. In all, 9 quartz particles and 59 carbon particles were found ingested by some 200 to 300 cells which were examined.

Since there were approximately twice as many carbon particles as quartz present, it may be concluded that carbon is ingested by sponge cells $\frac{59}{9 \times 2}$ or 3 times as readily as quartz. This agrees well with the results with leucocytes. The sponge cells were not considered sufficiently phagocytic to justify more quantitative experiments.

DISCUSSION

Whatever hypothesis is adopted to explain the more rapid ingestion of carbon, it must be concluded that there is for some reason greater attraction between the cell substance and the carbon than between the cell substance and the quartz. In view of the high adsorptive capacity of carbon this is not surprising, nor is this difference between different kinds of solid particles without its parallel in inorganic systems. Similar examples of selective "wetting" of solid particles are found in the flotation processes for the separation of different kinds of ores (8), which depend upon the fact that, in general, particles of the heavy metals have a greater affinity for oil and air phases than for water, and are thus floated to the surface while the worthless gangue particles such as silicates remain in the water and sink.

Rhumbler (9) remarks that the rhizopod *Euglypha*, in forming its shell of small particles, is able to distinguish between different materials. In imitating this he rubbed up coal and quartz particles in various oils and sprayed them into 70 per cent alcohol. In such instances the coal remains on the inside of the oil drops and the quartz collects in the surface. This observation being of considerable interest for these experiments has been repeated quantitatively with oil drops in 70 per cent alcohol and chloroform drops in water. In all cases the carbon collects in the interface between the phases more rapidly than the quartz.

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The results with chloroform are plotted in Fig. 12. Similar results are obtained with cottonseed oil drops in 70 per cent alcohol or water. Moreover, if a mixture of equal numbers of quartz and carbon particles in water is warmed up with phenol above the critical solution tempera-

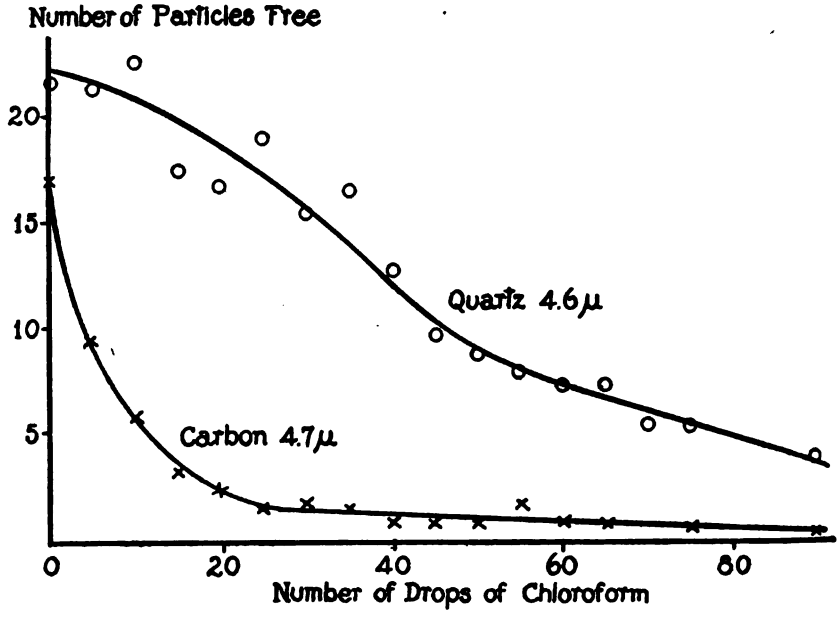

FIG. 12. Analogy to selective phagocytosis of carbon in a chloroform-water system. To 2 cc. of a suspension of carbon particles 4.7μ in diameter and quartz particles 4.6μ in diameter, chloroform is added 5 drops at a time. After each addition the mixture is shaken vigorously, allowed to stand a few minutes until the chloroform drops have settled to the bottom, and counts made of the number of carbon and quartz particles left. These figures are plotted as ordinates against the number of chloroform drops added as abscissae. Both particles accumulate in the chloroform-water surfaces, the carbon, however, much more rapidly than the quartz.

ture, on subsequently cooling the mixture, the majority of the carbon is found in the phenol phase and the majority of the quartz in the water phase, as shown by actual counts of the number of particles of each in the two phases, as follows:

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	In phenol phase.	In water phase.
Carbon	44	5
	60	20
Quartz	5	40
	2	9

ve qualitative experiments of a similar nature have been out by Reinders (10) and by Hofmann (11). Both agree .rticles of carbon are among those most easily adhering to ; of oil, chloroform ether, etc., but neither used quartz particles parison.

theoretical distribution of solid particles between two liquid according to the surface tension relations has been excellently rized by Thompson (12). In a general way it may be said a carbon-oil-water system the lower the potential energy of n-oil surface, the greater the chance of the carbon being in the *vice versa*. By collecting in the interface the oil-water surface :ed. Therefore, the higher the oil-water tension, other quan- eing equal, the greater the chance of the carbon being in the ry. By collecting in the interface the carbon decreases the al energy of the oil-water surface. This is presumably the for the emulsifying power of lampblack on water-kerosene ; reported by Moore (13).

)ugh the more rapid ingestion of carbon by leucocytes could predicted from the selective wetting of carbon by oil, phenol, loroform drops[3] the same principles of surface tension which the inorganic phenomenon can be applied to an explanation)iological fact. It may, therefore, be argued that carbon would :n up by leucocytes more rapidly than quartz, if the potential of a carbon-serum surface were greater than the potential

fallacy of predicting a surface tension of a particle in one medium from .ce tension in another is shown convincingly by the behavior of particles ganese dioxide and manganese silicate. The former are taken up less than the latter by drops of chloroform and paraffin oil, but about twice ly by sponge cells and in some experiments at least 20 times as rapidly eucocytes.

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energy of a quartz-serum surface, *other things being equal.* It is interesting that this is exactly the condition which should also cause more rapid clumping of the carbon than the quartz, since clumping involves a decrease of surface. It has already been mentioned that this difference in stability is the one outstanding distinction between quartz and carbon.[4]

According to Bredig (14) a low surface tension between a particle and a liquid should correspond to a high electric charge, thus reconciling electrical and surface tension theories of coagulation. If this theory is correct quartz should carry a higher charge than carbon, and the available evidence in the literature indicates that this is so. Thus, Whitney and Blake (15) measured the charge on quartz particles by kataphoresis and found a rather high negative charge of 0.042 volts. Rona and Michaelis (16) found that H and OH ions were equally adsorbed by carbon, and concluded, in consequence, that carbon must carry no electric charge. This opinion was, moreover, corroborated by a statement of Freundlich's (which they quote in a footnote) to the effect that this lack of a charge on carbon accounts for difficulty which he had experienced in trying to measure the kataphoresis of carbon. Rona and Gyorgy (17) were led to the same postulate concerning carbon by measurements of the effect of non-electrolytes on the speed of settling.

Some attempts were made to confirm this difference between the electric charges on quartz and carbon qualitatively by measuring the stability of the suspensions in various concentrations of sodium hydroxide and hydrochloric acid. Uniform suspensions, 2 to 3 microns in diameter, of quartz and carbon were used, and the degree of coagulation in the different solutions was determined by counts of the total number of particles, aggregate and single, in the case of carbon, and of the number of clumps in the case of quartz. The coagulation of quartz was so slight even in the most effective concentration of hydrochloric acid that there was scarcely a measurable decrease in the total number of particles. Both suspensions showed greatest stability in neutral and alkaline solutions and both showed an

[4] In agreement with this hypothesis is the fact that manganese dioxide is ingested by leucocytes with extraordinary rapidity compared to manganese silicate, and is also much less stable in suspension.

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ı coagulation between 0.0003 and 0.005 M hydrochloric acid
y at 0.001 in the case of quartz). The results, therefore,
prove nor disprove the hypothesis although they do indicate
bon carries a negative charge contrary to the evidence from
ature. Also, the greater stability of quartz as compared to
in all concentrations of hydrochloric acid appears to prove
bility (and hence probably phagocytosis) is conditioned by
ctor besides the electric charge, presumably the surface

are many valid objections (McClendon (18), Hyman (19),
b (20)) to the explanation of ameboid movement by surface
changes on account of the more or less rigid ectoplasm of the
ı so far as phagocytosis is limited by ameboid movement,
jections also apply to the explanation of ingestion of particles
ıce tension. The ingestion of quartz in these experiments,
', is not limited by the ameboid movement of the leucocytes
; sufficiently vigorous to cause ingestion of carbon at a more
ıte. It is, therefore, a question of molecular attractions
. particle and cell, and these are best expressed in terms of
tension.

ınalogy between this hypothesis and the sometimes parallel
)f opsonins and agglutinins has been suggested to the writer
W. B. Cannon. Thus, Bull (21) in a series of papers has
. out the fact that bacteria injected into the circulation are
l more rapidly, apparently by phagocytosis, when agglutinated
ıune serum. Zinsser (22) quotes Ottenberg as authority for
ement that phagocytosis of foreign red cells in the circulation
ood transfusion occurs only when the patient's serum has an
ıative action on the donor's cells. Likewise, Ledingham (23)
nted out "that the opsonin-bacteria like the agglutinin-bac-
: probably in a more precipitable condition than non-sensitized
ı. They are, in fact, as I have frequently observed, in a con-
)f extremely fine aggregation, and if the sensitizing fluid has
agglutinating powers the sensitized organisms are frankly
l." From this evidence it appears that bacteria as well as
ırticles are more readily ingested when easily agglutinated.

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SUMMARY

1. The rates of ingestion of quartz and carbon particles by leucocytes, when both are in suspension in serum, was compared with the availability of the two particles as predicted from the calculated chances of collision with the leucocytes, and it was shown that carbon is ingested about 4 times as readily as quartz.

2. The greater ease of ingestion of carbon was verified by a new method of measuring phagocytosis, described as the film method in which the cells ingest particles as they creep about on a slide.

3. The relative rates of ingestion of carbon and quartz depend upon the condition of the cells, the difference increasing as the phagocytic activity of the cells decreases.

4. Sponge cells also ingest carbon about 3 times as readily as quartz.

5. The hypothesis is suggested that the cause of the more rapid ingestion of carbon may be identical with the cause of the greater instability of the carbon suspensions.

6. An inorganic analogy to this selective phagocytic action is offered.

7. The application to opsonins and agglutinins is discussed.

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STUDIES ON ENZYME ACTION.

XIX. The Sucrolytic Actions of Bananas.

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INTRODUCTION.

hange of starch into simpler carbohydrates such as glucose, etc., or the reverse reaction, is a general phenomenon which anies the growth and development (so called ripening) of lible fruits and also of a number of vegetables. For example, ch of apples and of bananas in the maturing of the fruit s converted into simpler carbohydrates, while the sugars of ernels and of peas are converted into starches.[1] In the belief zymes and enzyme actions play an important, if not a pre- ting, rôle in these chemical changes, a study was begun of the s which might be expected to be involved in these reactions. ıana (*Musa sapientum*) was chosen as a typical example of a which such changes occur because of the possibility of readily ıg large quantities of the unripe fruit, the rapidity with which ıe made to ripen, the fact that it ripens when separated from ving plant, and the extensive change from starch into glucose, ıugar, and sucrose which accompanies the ripening.

Previous Work.

the purpose in view, the chemical composition of the unripe e bananas is of interest. The results given by Gore[2] are per- ıe most satisfactory and may be quoted as follows: As a result ıing a number of bananas, a loss in weight of 3.88 per cent was

[1] herman, H. C., Chemistry of food and nutrition, New York, 1918, 12.
[2] , H. C., *J. Agric. Research*, 1914–15, iii, 187.

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observed. When unripe, the fruit consisted of 41.72 per cent peel and 58.28 per cent pulp; when ripe, 37.85 per cent peel and 62.15 per cent pulp. During ripening, the main changes in the peel consisted of a decrease of 5 per cent in water content and the transformation of two-thirds of the starch into sugar. In the pulp, the starch content changed from 13.15 to 2.40 per cent, the reducing sugars from 0.37 to 10.34 per cent, and the sucrose from 0.38 to 1.52 per cent, while the water content increased 1.6 per cent.

Enzyme studies on bananas have been carried out from time to time. The most complete investigations were published by Tallarico,[3] who reported the presence of sucrase, amylase, protease, and catalase, the absence of lipase, while the results for tyrosinase were not conclusive, and by Bailey,[4] who found amylase, sucrase, raffinase, protease, lipase, and peroxidase, but not maltase, dextrinase, or lactase. Sucrase and amylase are mainly of interest in connection with the present work. Both Tallarico and Bailey proved qualitatively the presence of sucrase in bananas, considerably more in ripe than in unripe ones. With regard to the amylase, the results were not so satisfactory. The errors in the method used by Tallarico apparently were as great as the observed actions. Bailey's results were obtained with the iodine test under certain conditions.

Methods of Testing.

The amount of amylase or of sucrase action was determined in most of the experiments by the estimation of the reducing sugars formed.[5,6] In carrying out the estimations, from 2 to 10 cc. portions of the mixtures tested were used, depending upon the amount of reducing substance present.

In a few experiments the starch-splitting actions were followed by adding iodine dissolved in potassium iodide solution to the mixtures.

Sodium hydroxide and hydrochloric acid solutions were employed to bring the mixtures to definite hydrogen ion concentrations. In

[3] Tallarico, G., *Arch. farm. sper. e sc. aff.*, 1908, vii, 27, 49.

[4] Bailey, E. M., *J. Am. Chem. Soc.*, 1912, xxxiv, 1706.

[5] Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1083.

[6] McGuire, G., and Falk, K. G., *J. Gen. Physiol.*, 1919–20, ii, 217.

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the series of sucrase actions, buffer mixtures were added
o the enzyme-substrate mixtures. The indicators recom-
y Clark and Lubs were used.[7]
number of different procedures were employed to obtain the
reparations from the bananas, these will be given in con-
ith the results obtained.

General Properties of Unripe and Ripe Bananas.

f the general properties of the unripe and ripe bananas[8] will
iven. The pulp of the unripe banana adheres to the peel,
eeling difficult, while with the ripe banana the peel is easily
The pulp of the green banana is more fibrous in character
es with difficulty, in marked contrast to that of the ripe
hich readily forms a wet, soft paste. On mixing with water,
of the former does not form a homogeneous mass, that of
does. In extracting (grinding) repeatedly with small por-
water and squeezing through muslin, a white cloudy liquid
rge quantity of insoluble residue are obtained from unripe
ulp, while from ripe banana pulp most of the mixture can be
through the muslin leaving only a small amount of very soft
The pulp of the unripe banana differs from that of the ripe
lso, in containing a sticky substance which discolors the

alitative factors which have been given, as well as the color
el, serve to show the state of ripeness of the banana. More
tive relations have been obtained by use of the "coefficient
ss" which represents the ratio of weight of pulp to weight of
he different stages.[3,4] This ratio increases in value as ripen-
eeds.[1] The decrease in the percentage of starch present
the peel or in the pulp might also be used as a quantitative
of the state of ripeness.

W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191.
ish to thank the Fruit Dispatch Company for supplying the greater
e bananas used in this investigation.

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Enzyme Tests with Unripe Bananas.

The enzyme tests with unripe bananas can be summarized briefly; since (*a*) the general relations found were the same as with ripe bananas, except that (*b*) greater enzyme actions were obtained with ripe banana preparations than with unripe, and (*c*) the nature and properties of the unripe banana rendered working with it more difficult and less satisfactory.

The methods of studying the unripe banana pulp were as follows:

1. Pulp extracted with equal weight of water, filtered through muslin. Filtrate and residue (to which an equal quantity of water had been added) tested at different hydrogen ion concentrations in 1 per cent sucrose (also more concentrated) and starch solutions for 18 hours at 35°C.

2. Same as Method 1 with one-half the weight of water.

3. Same as Method 1 with one-quarter the weight of water kept between 5° and 10°C. during the preparation.

4. Pulp ground in food chopper, no water added.

5. Pulp and peel ground together in food chopper, no water added, and paste tested (mainly for amylase).

6. Pulp ground with twice its weight of 95 per cent ethyl alcohol, filtered through heavy muslin, and centrifuged. Residue ground with half its weight of water, filtered through muslin, centrifuged, and residue and liquid tested.

It may be pointed out here that upon the addition of Lintner starch solution to unripe banana extract, a precipitate was immediately formed. With ripe banana extracts there was no precipitate. With the unripe extract filtered through paper, upon the addition of Fehling's solution to determine the enzyme actions, a gelatinous precipitate was formed. Only small portions could therefore be used for the tests as otherwise filtration through the Gooch crucibles was difficult. No such difficulty was experienced with ripe banana extracts. Toluene was used as preservative throughout these experiments.

The general results of the enzyme tests were as follows: For amylase action, in no case were the results such as to show definitely the presence of this enzyme. In a few experiments small apparent

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were observed, but, in view of possible experimental errors, parent actions were not of sufficient magnitude to prove ely the presence of such an enzyme. The object of the ary treatment with alcohol (Method 6) was for the purpose lving possible inhibiting substances, such as tannin, with ıltaneous precipitation of the enzyme in order to obtain er separated from inactivating soluble material. The sucrase were positive, considerable action being obtained. ripe banana could be handled more readily, a more extended ntitative examination of the sucrase from this source will be

of the results with the unripe banana preparations are given the nature of the actions. 20 gm. of pulp obtained by 4 with 10 cc. of 2 per cent starch solution after 21 hours at ıve reducing substances corresponding to 5.0 mg. of Cu_2O of pulp, and with 10 cc. of 20 per cent sucrose solution simi- e reducing substances corresponding to 246 mg. of Cu_2O per ulp. 20 gm. of pulp plus peel mixture obtained by Method 5 similarly gave with the starch no reducing substances, and sucrose reducing substances corresponding to 203 mg. of r gm. of pulp plus peel.

esults of Bailey[4] on the action of air on the green banana pulp ıfirmed, no amylase being obtained by this treatment.

Soluble Sucrase Preparation from Ripe Bananas.

e and insoluble sucrase preparations were obtained from ripe . As a result of a number of different methods of extrac- following procedure was found to give the most satisfactory preparation. The banana pulp was ground rapidly in a food , the finest cutter being used, then mashed in a porcelain with a wooden potato masher with normal sodium chloride (100 cc. for each 400 gm. of pulp). Toluene was added and ture then filtered through paper. The filtrate was dialyzed hours in collodion bags against running water to remove soluble dialyzable carbohydrates, and other products. The solution was used in the sucrase experiments.

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A number of experiments were carried out in which the treated pulp was centrifuged and the supernatant liquid dialyzed and used, or where the treated pulp was squeezed through muslin and then centrifuged. The properties of the sucrase solutions so obtained were essentially the same, but the above procedure was the one finally adopted for studying the soluble banana sucrase.

The following methods of extraction did not yield preparations so satisfactory as the salt treatment described: Extraction with one-fourth weight or equal weight of water (fairly active preparations, in one case 2½ times as much action was obtained by the salt extraction as by the water extraction); extraction of small portions of banana with ten times the weight of water or salt solution; autolysis, followed by filtration, etc., as in the preparation of yeast sucrase[9] (slightly active filtrate obtained); and grinding in a ball mill for a long period of time (inactive filtrate).

It may be mentioned that repeated extractions did not offer any appreciable advantage over a single treatment. A small amount of active material could be obtained on a second extraction as compared with the first extract. Extracting for longer periods of time did not give appreciably more active solutions.

The following results were obtained in testing at different hydrogen ion concentrations. 10 cc. of the salt-extracted dialyzed solution plus 10 cc. of a 20 per cent sucrase solution were incubated for 2½ hours at 35°C. The actions are given in terms of mg. of Cu_2O produced by the action of 1.0 cc. of original undialyzed filtrate corrected for blanks.

pH	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5
Actions	183	481	489	478	458	375	135	52

Fig. 1 shows these results graphically. There is a zone of maximum action between pH 3.5 and 4.5, with rapid drops beginning between 3.5 and 3.0 and at 5.0. This optimum zone corresponds to that observed with yeast sucrase solutions, where the zone is of different widths in various experiments and under different conditions,[10] and with

[9] Nelson, J. M., and Born, S., *J. Am. Chem. Soc.*, 1914, xxxvi, 393.

[10] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxv, 386. Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2769. Michaelis, L., and Rothstein, M., *Biochem. Z.*, 1920, cx, 217.

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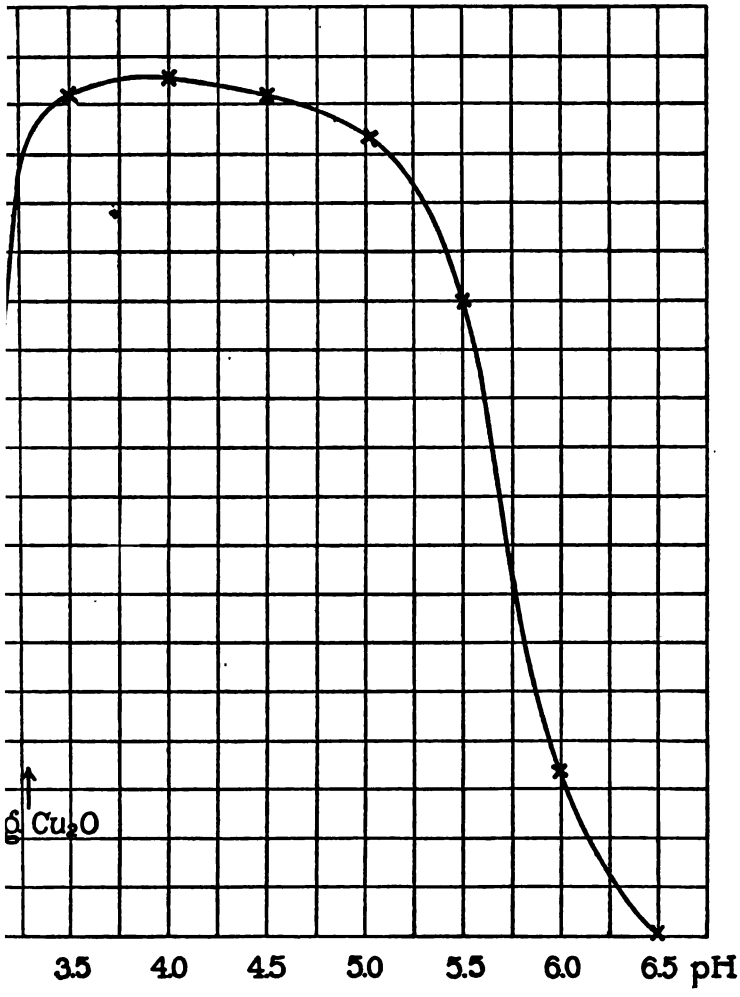
ıcrase.[6] The exact optimum pH is difficult to determine ıse because of the flat portion of the curve, but with soluble



Action of soluble banana sucrase preparation at different hydrogen ion ıtions on sucrose.

sucrase it is not far removed from 4.0 at 35°C. for 2½ hours The method of presenting these results is not the most tory but it shows definitely the optimum conditions. Not

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enough data were obtained to calculate the velocity constants satisfactorily, assuming that such constants would be obtained. In order to determine the different times for the same action, further assumptions would have to be introduced. The hydrogen ion concentrations of the solutions did not change during the actions.

The amounts of sucrase action with the same quantity of enzyme material at different intervals of time were studied. The results are given in terms of mg. of Cu_2O obtained, calculated back to 1 cc. of original extract with 2.5 and 5 per cent sucrose solutions. The hydrogen ion concentration was that of the natural juice, not far removed from the optimum.

Time (hrs.)	1	2	3	4	5	6	24
2.5 per cent sucrose solution	65	134	210	224	228	218	230
5.0 " " " "	72	150	226	319	394	395	393

These results are shown graphically in Fig. 2. The one point at 6 hours with 2.5 per cent sucrose solution is evidently incorrect. The experimental errors are magnified by the calculations, since the difference indicated by 228 and 218 was caused by a difference in the weighings of 2.3 mg. of Cu_2O.

These results bring out the following facts. The action is a linear function of the time, the amounts hydrolyzed being proportional to the time of action until practically all the sucrose was hydrolyzed.[11] The action with 5 per cent sucrose solution was only slightly greater than with the 2.5 per cent solution, indicating that the sucrase was nearly saturated in the more dilute solution. However, the total action for the more concentrated solution was not twice as large as for the dilute solution in the limited times used. The products of the reaction evidently played a part here, interfering with the action of the sucrase. These results are similar to those obtained in the more extended investigations of yeast sucrase by others.

Active precipitates were obtained by the addition of alcohol or of acetone to the sucrase solution. These did not form clear solutions again, but showed considerable actions when tested as suspensions.

[11] Similar relations within certain limits have been observed with yeast sucrase. Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790. Michaelis and Rothstein.[10]

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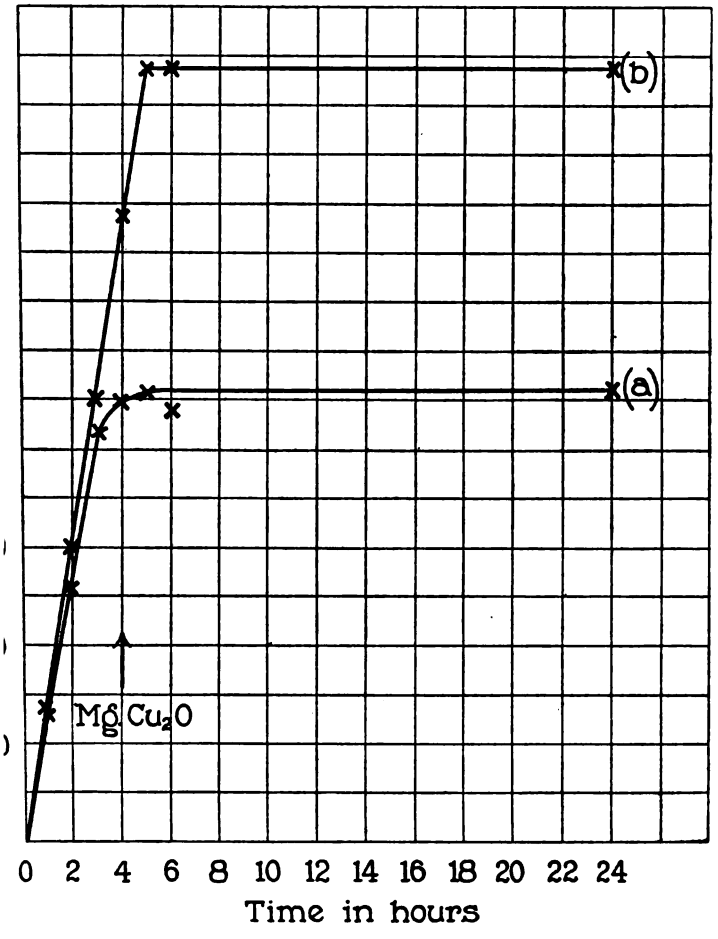
ntinued treatment with alcohol appeared to inactivate the These preparations were not studied further since the con-



2. Time-action curves of soluble sucrase preparation; (*a*) 2.5 per cent and per cent sucrose solutions.

1 of soluble into insoluble sucrase preparation in a different ppeared to be of more direct interest.

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Insoluble Sucrase Preparation from Ripe Bananas.

It was found that the water (or salt solution) insoluble residue from the ripe banana pulp possessed considerable sucrose-hydrolyzing action.[12]

2,760 gm. of pulp were ground with 700 cc. of N sodium chloride solution, 16 liters of water added, thoroughly stirred, allowed to stand over night at room temperature, and filtered through paper. The moist residue was treated with 10 liters of water similarly, and the residue (drained more thoroughly than the first residue) treated again with 6 liters of water and then with 8 liters. The filtrate from the last gave no sucrase action. The residue was dried by grinding twice with alcohol and then with ether. 0.75 gm. of this residue tested in 20 cc. of a 10 per cent sucrose solution gave reducing substances in 4 hours at 35°C. producing 355 mg. of Cu_2O per 0.10 gm. of residue.

The possibility that the cell walls of the banana pulp were not broken and that the sucrase was retained within the cells was tested a number of times by using different methods of grinding and extracting. Vigorous grinding in a mortar with sand as well as long continued grinding in a ball mill, also followed in some cases by grinding in a mortar, gave, after thorough extraction, active insoluble residues. In place of filtering through paper, the residue was also obtained by centrifuging or by filtering through asbestos, and showed similar actions. It was obtained as a grayish brown powder by grinding twice with 95 per cent alcohol, separating by centrifuging, and finally washing with ether and drying on filter paper at room temperature. This residue gave a nitrogen content of nearly 1 per cent.

A series of determinations with the centrifuged moist residue at different hydrogen ion concentrations gave an optimum action between pH 4.0 and 4.5 with a rapid falling off beyond 6.0. In view of the character of the material, a more careful determination was not made. The results showed an optimum similar to that of the soluble sucrase preparation which was the main question involved.

[12] Euler and Svanberg (Euler, H., and Svanberg, O., *Z. physiol. Chem.*, cvii, 269) recently studied the sucrase actions of the residue from autolyzed and extracted yeast.

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Conversion of Soluble into Insoluble Sucrase Preparation.

The finding of soluble and insoluble sucrase preparations or materials showing sucrase actions led to further developments. The optimum action for both was found to be at approximately the same pH (nearly 4.0). This raises the question, which was developed at some length in another connection,[13] as to whether a definite enzyme action was connected with a certain molecule or with a certain group which may be present in different molecules. If the latter view is adopted tentatively, the same active sucrase grouping would be present in the one case in a soluble molecular species, and in the other case in an insoluble molecular species. This point of view simplifies in some ways the consideration of the experiments to be described in this section.

That sucrase-carrying substances of different solubilities are present in banana pulp is shown by the fact that aqueous extraction yields one such, normal sodium chloride solution extraction following the aqueous extraction another, and the residue after repeated extraction a third. To judge from the hydrogen ion concentration for optimum action these sucrase actions are identical.

The most active and satisfactory soluble preparation was obtained by the salt extraction as described. The sodium chloride and the soluble simple sugars, etc., were removed by dialyzing over night in collodion bags against running tap water. The volume increased as a rule about 60 to 80 per cent. If the dialyzed sucrase solution was further dialyzed in a fresh collodion bag for 24 hours against tap water, the volume did not increase but a gel separated. The hydrogen ion concentration of the mixture did not change in the course of dialysis on the 2nd day. The gel could be filtered out readily by means of filter paper. The filtrate from the gel did not show sucrase action but the gel showed very marked activity. The gel was ground with alcohol twice and ether once and allowed to dry in the air at room temperature. It did not dissolve in water, but formed a gel with it. A suspension showed the following activity: 0.05 gm. of material in 20 cc. of a 10 per cent sucrose solution in 4 hours at 35°C. gave reducing substances producing 457 mg. of Cu_2O per 0.01 gm. of

[13] Falk, K. G., The chemistry of enzyme actions, New York, 1921, 81.

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solid preparation. It was therefore about fifteen times as active as the insoluble sucrase preparation obtained directly from the banana pulp. It showed a nitrogen content of 4.4 per cent; that is, about four times as large as that of the latter.

Amylase Results with Ripe Bananas.

The amylase tests carried out with Lintner starch or with banana starch, in which the amounts of reducing sugars were determined, did not give definite evidence of the presence of a starch-splitting enzyme. These tests were carried out with extracts and suspensions prepared as described in the sucrase experiments. Before determining the amounts of reducing sugars formed, the mixtures were filtered through paper and an aliquot of the filtrate taken. Undialyzed extracts gave considerable blanks which increased on incubation because of the sucrase action on the sucrose contained in the banana. Dialyzed extracts gave little or no blanks. Isolated results at times showed an apparent action, but the results were not consistent enough or large enough to warrant the statement of a definite saccharogenic action on the starch. The mixtures were tested at different hydrogen ion concentrations and also in the presence of salt. The solid residue from the extractions behaved similarly.

In view of Bailey's positive results with the iodine test[4] (amyloclastic in place of saccharogenic actions) and since he found more marked actions with ripe than with unripe banana pulp, the reaction was studied in this way also. The method described by him was followed as closely as possible. It was found that, with the suspensions of banana pulp on incubation with Lintner starch, precipitates settled to the bottom of the tubes; that if these mixtures after incubation were tested with iodine after filtration, the filtrate gave no starch reaction but the precipitate on the paper became blue; that, if the whole mixture without filtering was shaken and tested with iodine, the precipitate was colored blue and settled leaving the supernatant liquid colorless. It is probable, though not altogether clear from the description of his experiments, that Bailey filtered or decanted these mixtures before testing with iodine. If that was the case, the amyloclastic actions which he described are open to ques-

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Repeating his work, using the most satisfactory method of
ıg active preparations which he described, as well as extracts
d as described with the sucrase experiments and incubating
per cent Lintner starch solution for 24 to 40 hours at 30° to
.t was found that no amyloclastic actions as marked as those
ed by Bailey were obtained. The greatest change which was
:d in any of the tests was a change from the deep blue iodine
:eaction to a bluish violet color test. The amyloclastic actions
ıerefore be said to be extremely small if present at all. In all
ests, toluene was used as preservative.

DISCUSSION.

change of soluble sucrase material into insoluble during the
treatment of dialysis raises the question of the state of the
ɜ in the growing banana. On grinding to a pulp and extract-
)luble and insoluble preparations were obtained. This treat-
may, however, have been sufficient to change materially the
:ties of the substances originally present. It is therefore con-
)le that the sucrase may be present as a completely soluble sub-
: in the ripening fruit and that the differences in solubility
/ed were due entirely to the treatment to which the fruit was
:ted. It must also be recalled that toluene was added when-
he treatment extended over a greater period of time than a few
:es and that this toluene may exert a definite influence, possibly
:oagulating nature, upon the substances present.

is change in solubility was also observed when the bananas were
ıd in a ball mill with toluene present. 8 hours grinding followed
:traction gave a certain amount of soluble sucrase although less
was obtained by grinding in a mortar for a shorter period of
Grinding for a week, however, resulted in the extract showing
ıcrase action at all. The residue showed marked activity. It
obable that here, too, the soluble sucrase was converted into
uble material. The reasons or causes for this change, whether
to dialysis and removal of certain products, the action of toluene,
ther cause, are not known but will be investigated further.

Cf. similar observations with potato amylase.[6]

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The change of soluble into insoluble sucrase upon the simple treatments described illustrates the sensitive character of materials occurring in living matter. In this case, the enzyme property is not destroyed but a different property (solubility), which can be traced by following the enzyme action, is changed.

Sucrase has been generally taken to be one of the most hardy of the enzymes. It is not inactivated as rapidly as most other enzymes or under conditions which cause these to lose their activity completely. On the other hand, amylase has been found to be highly sensitive to outside influences and to be destroyed under comparatively simple conditions.[15] It is possible that the simple treatments, which changed the properties of the material carrying the sucrase action without destroying that action, may destroy the amylase action which would be expected to be present in the ripening banana. Such an explanation would account for the failure to obtain a definite and marked amylase action in banana pulp preparations or extracts.

The question of the conversion of starch into simpler carbohydrates may be considered further. For such an action in living, growing matter, the presence of the enzyme amylase, on the basis of past experience, is required. Experimental tests have not shown conclusively that this enzyme is present in the banana. Reasoning on the basis of the active amylase preparations described by Sherman[15] a minute quantity of such material would suffice to produce the changes observed in the banana, but, on the other hand, it should be possible to obtain experimental evidence of the presence of such an enzyme. It is also possible that substances are present in the banana which when brought into close contact, as by grinding, with the enzyme material, inhibit the action. It is possible to imagine a cellular structure of such nature that enzyme material and inactivating substance (possibly tannin) are separated in the fruit in the form and shape in which it occurs in nature and that artificial treatment involving destruction of the cell structure is accompanied or followed by profound changes in the cellular contents.

There is, however, another possible view-point. In ripening "The most conspicuous change is the long-recognized conversion of

[15] Sherman, H. C., and Schlesinger, M., *J. Am. Chem. Soc.*, 1915, xxxvii, 1305.

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starch into sugars. It is most rapid while the fruits are turning from green to yellow. During this period the respiration rate increases manyfold, becoming greatest at the time when the rate of starch hydrolysis is most rapid."[2] Bailey[16] showed that the banana ripened in the presence of oxygen, but not in gases such as hydrogen, carbon dioxide, etc. It is therefore possible that the ripening process, or the breaking down of the starch, is not merely an amyloclastic action as this is commonly understood, but involves a simultaneous or preliminary oxidation reaction.

SUMMARY.

A number of different methods of treatment of unripe and ripe bananas for the purpose of obtaining and studying sucrolytic and amylolytic enzymes are described.

No conclusive evidence of the presence of an amlyase could be obtained in any of the preparations.

The sucrase of unripe and ripe bananas was studied more extensively. With ripe bananas, both soluble and insoluble sucrase preparations were obtained. Conditions for converting the soluble into an insoluble form were found. The actions of the sucrase preparations as far as the hydrogen ion concentration for maximum action and the time-action relation are concerned are similar to the behavior of the yeast and the potato sucrase.

[16] Bailey, E. M., *J. Biol. Chem.*, 1905–06, i, 355.

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A THEORY OF INJURY AND RECOVERY.

III. Repeated Exposures to Toxic Solutions.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, March 21, 1921.)

In previous papers a theory is developed which enables us to predict the behavior of *Laminaria* when transferred from sea water to toxic solutions and then replaced in sea water. If the theory is sound it should also enable us to predict the behavior of tissue transferred from one toxic solution to another. In order to put this to a test a variety of experiments was made in which the tissue was exposed to several solutions in succession.

The experiments and calculations were carried out as previously described.

I. Alternate Exposure to NaCl and Sea Water.

The procedure may be illustrated by a typical experiment, the results of which are shown in Fig. 1.

The tissue was exposed for 20.8 minutes[1] to 0.278 M NaCl, during which time the resistance fell from 100 to 74.03 per cent. The tissue was then replaced in sea water. The resistance at any time, T_R, after this replacement may be calculated by means of a formula which has been explained in a previous paper.[2] The formula may be expressed as follows:

$$\text{Resistance} = (A+S)\left(\frac{K_A}{K_M-K_A}\right)\left(e^{-K_A T_R}-e^{-K_M T_R}\right)+Me^{-K_M T_R}+L\left(\frac{O}{90}\right)+10. \quad (1)$$

[1] This is corrected from 20 minutes (as explained in a previous paper[2]) in order to make it conform to the standard curve. An example of this is given in the present paper in discussing the change of resistance which occurs during the second exposure to NaCl.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 145.

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in which $K_A = 0.0036$, $K_M = 0.1080$ and M is equal to the observed resistance at the moment when the tissue is replaced in sea water less 10 (since the base line of the curve is taken as 10).

The value of A may be obtained by the formula

$$A = 2700\, e^{-K_A T_E}. \tag{2}$$

in which $K_A = 0.018$ and T_E is the time of exposure to NaCl (in this case 20.8 minutes).

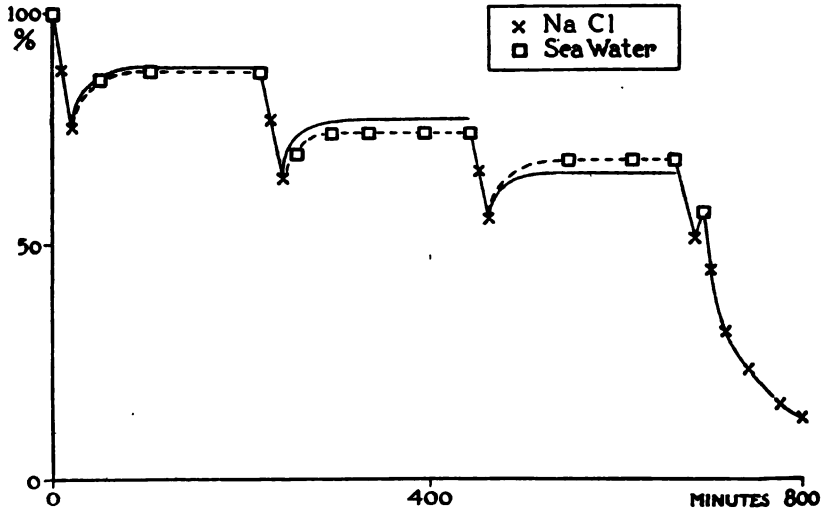

FIG. 1. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M and in sea water. Unbroken line, calculated values; broken line, observed values (at the extreme right these coincide). Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

The value of S may be obtained by the formula

$$S = 1041.77\left(\frac{K_R}{K_S - K_R}\right)\left(e^{-K_R T_E} - e^{-K_S T_E}\right) + 2.7\, e^{-K_S T_E} \tag{3}$$

in which $K_R = 0.04998$ and $K_S = 0.02856$.

The value of O may be obtained by means of the formula

$$O = 89.1\left(\frac{K_N}{K_O - K_N}\right)\left(e^{-K_N T_E} - e^{-K_O T_E}\right) + 90\, e^{-K_O T_E} \tag{4}$$

in which $K_M = 0.03$ and $K_O = 0.0297$.

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The value of L may be obtained by means of the formula

$$L = 100 - \left[2700\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T_R} - e^{-K_M T_R}\right) + 90\, e^{-K_M T_R} + 10\right] \quad (5)$$

in which $K_A = 0.0036$ and $K_M = 0.1080$ (these are the normal values in sea water).

After 20.8 minutes in the solution of NaCl the value of T_E (in formulas (2), (3), and (4)) is 20.8 and the following results are obtained: $A = 1856.80$, $S = 484.06$, $M = 64.03$, $O = 88.41$. When the tissue has been replaced in sea water and left for 10.4 minutes[3] the value of T_R (in formulas (1) and (5)) is 10.4 and the value of L is found to be 1.33.

Substituting these values in formula (1) we find that when the tissue has been replaced in sea water the resistance at the end of 10.4 minutes is 83.49. Proceeding in this manner we calculate the resistance at various intervals after replacement in sea water and obtain the first (calculated) recovery curve shown in Fig. 1. It is evident that it is in fairly good agreement with the observed values.

After 200 minutes in sea water (during which the resistance rose to 87.10 per cent and remained practically constant) the tissue was replaced in the solution of NaCl. In the course of 21.2 minutes[4] the resistance fell from 87.10 to 64.18. It was then replaced in sea water. The recovery curve may be calculated as before, the only differences being as follows:

1. On replacing the tissue in sea water the destruction of O (by the reactions $N \rightarrow O \rightarrow P$) ceases (or becomes negligible); hence the value of O at the beginning of the second exposure (if equilibrium has been reached) is that of the observed resistance less 10, or $87.10 - 10 = 77.10$. We find by means of formula (4) that when O at the start equals 90 it loses 11.95 during an exposure of 21.2 minutes to the solution of NaCl, but as it only equals 77.10 at the start the loss will be $11.95\ (77.10 \div 90) = 10.23$. Subtracting this from 77.10 gives 66.87, the value of O at the end of the second exposure, and adding 10 (since the base line is 10) makes 76.87, the level to which the resistance should rise after the second exposure.

[3] This is corrected from 10 minutes[1] as explained in a former paper.

[4] The actual time was 20 minutes: the manner in which the corrected figure is obtained is explained in a subsequent paragraph.[1]

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2. At the start of the first recovery[5] S is rapidly converted into A but is partially restored during the subsequent stay in sea water and at the beginning of the second exposure equals 2.7 $(O \div 90)$ in which O has the value given above (77.10).

3. During exposure to NaCl the value of R diminishes from R_0 to R_1 according to the formula

$$R_1 = R_0 e^{-K_R T_E} = 1041.77\ e^{-(0.0498)42} \qquad (6)$$

in which R_0 = the value of R before the first exposure (1041.77) and T_E equals the total exposure to NaCl (20.8 + 21.2 = 42).

It is evident that unless R is restored during the period in sea water the speed of recovery will fall off somewhat with each successive exposure.

4. The value of M is the observed resistance (at the end of the second exposure) less 10 or 64.18 − 10 = 54.18.

5. The value of A is obtained by multiplying by 30 the resistance observed at equilibrium (less 10). This is based upon the following considerations:

Just before the beginning of the second exposure A and M are assumed to be in equilibrium in sea water, in which case as much of A must decompose in any minute as of M (otherwise M would not remain constant). But the amount of A which decomposes in 1 minute is AK_A and of M is MK_M; and since K_M is 30 times as great as K_A it follows that $A = 30M$. At the beginning of the second exposure $M = 87.10 - 10 = 77.10$ and $A = (77.10)30 = 2313$.

In order to ascertain how the resistance would change during the second exposure if it conformed to the standard curve employed in a previous paper[2] we may employ the formula

$$\text{Resistance} = 2313\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T_E} - e^{-K_M T_E}\right) + 77.1\ e^{-K_M T_E} + 10 \qquad (7)$$

in which $K_A = 0.018$, $K_M = 0.540$ and T_E = time the tissue has remained in the solution of NaCl. Comparing the values thus obtained with the observed resistance after an exposure of 20 minutes

[5] If the value of O were 90, S would be completely restored to its original value of 2.7 but since O has fallen to 77.10 it can only restore S to 2.7 (77.10 ÷ 90).

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we find that if the time is multiplied by 1.06 (making it 21.2 minutes) the observed resistance (64.18) agrees with the standard curve. This figure is therefore adopted. The value of T_E in formulas (2), (3) and (4) should now correspond to the total exposure to NaCl, and is 20.8 + 21.2 = 42.

These data were employed in calculating the second recovery curve and the results are shown in Fig. 1. The third recovery curve was calculated in the same fashion.

Instead of waiting for the establishment of equilibrium we may replace the tissue in NaCl after it has been for a short time in sea water. During the fourth recovery, after the tissue had been 10.2 minutes in sea water and the resistance had risen to 54.92 per cent it was replaced in sea water: the subsequent fall in resistance was calculated by means of formula (7). For the value 77.1 in this formula we must substitute the observed resistance less 10, or 55.89 − 10 = 45.89; and in place of 2313 we must substitute the present value of A. We assume that at the beginning of the fourth exposure to NaCl equilibrium had been reached in sea water: hence as the resistance was 68.10 the value of A (which we may call A_1) is, $A_1 = 30(68.10-10)$. During the fourth exposure to NaCl (lasting 20.4 minutes) the value of A_1 diminished to A_2 according to the formula

$$A_1 e^{-(0.018)20.4} = A_2$$

On replacing the tissue in sea water A_2 was augmented by the conversion of S into A. The value of S is found according to formula (3) in which T_E is equal to the total time of exposure (20.8 + 21.2 + 20.8 + 20.4 = 83.2). We may call this S_1. Hence the value of A immediately after replacement in sea water is $A_3 = A_2 + S_1$. During the subsequent 10.2 minutes in sea water A_3 diminished to A_4 according to the formula

$$A_3 e^{-(0.0036)10.2} = A_4$$

But at the same time it received an addition from the decomposition of O; the amount of this may be found as follows: The loss of A in sea water under normal conditions[6] in 10.2 minutes is

[6] The principle upon which this formula is based is explained in a previous paper in discussing the loss of M and its replacement by O. In the present case the effect of S is negligible since the amount of S in sea water is only 2.7.

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$$\text{Loss} = 2700 - \left(2700e^{-(0.0036)10.2} \right) = 97.26 \qquad (8)$$

and this could be completely replaced by O if O were intact. But since O has diminished[7] from 90 to 50.86 it can supply only $97.26(50.86 \div 90) = 54.95$. This must be added to A giving $A_5 = A_4 + 54.95$. The value of A_5 must be substituted for 2313 in formula (7). This enables us to calculate the fall of resistance after the last recovery (of 10.2 minutes). Fig. 1 shows the values so obtained and also the observed values.

II. Alternate Exposure to $CaCl_2$ and Sea Water.

When the tissue of *Laminaria* is transferred from sea water to a solution of $CaCl_2$ (of the same conductivity as sea water) the resistance rises and then falls as shown in Fig. 2. When it is replaced in sea water the resistance falls (much more rapidly than if left in the solution of $CaCl_2$) and eventually becomes stationary. This fall of resistance may be spoken of as recovery, since it may be regarded as analagous to the rise of resistance which occurs when tissue is transferred from NaCl to sea water.

Recovery after exposure to $CaCl_2$ may be calculated in precisely the same manner as recovery after exposure to NaCl. The only difference is that in formulas (2), (3), (4), (6), and (7) we must employ for the velocity constants (K_N, K_O, K_R, K_S, K_A and K_M) the values given for $CaCl_2$ in Table II of the preceding paper.[8] In formulas (1) and (5) the values of the velocity constants are always the same ($K_A = 0.0036$ and $K_M = 0.1080$) since these are the values which are normal for sea water.

Results of such calculations are shown in Fig. 2 together with the observed values.

[7] This is calculated as follows: At the beginning of the fourth exposure $O = 68.10 - 10 = 58.10$. If its value were 90 it would lose 11.23 during an exposure of 20.4 minutes to NaCl. Since $O = 58.10$ the loss will be $11.23\ (58.10 \div 90) = 7.24$: subtracting this from 58.10 we have 50.86.

[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 415.

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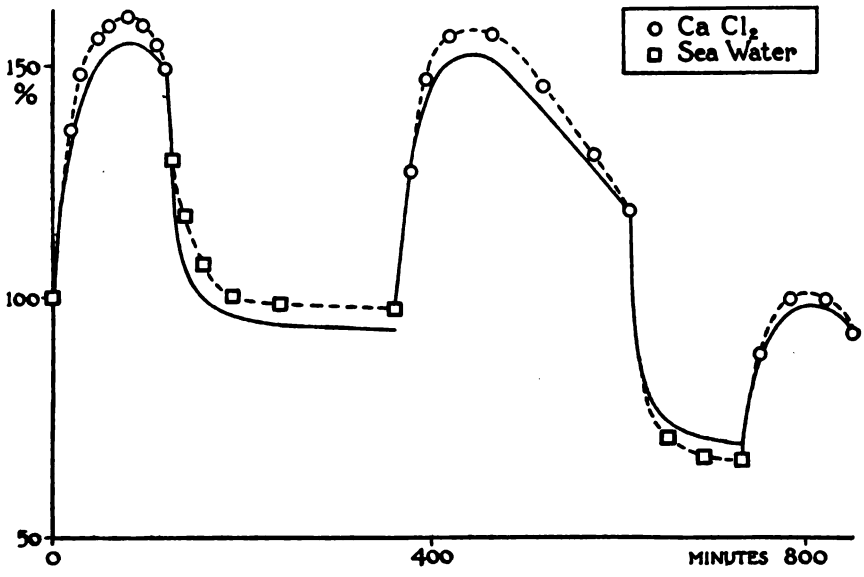

FIG. 2. Curves showing the electrical resistance of *Laminaria agardhii* in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

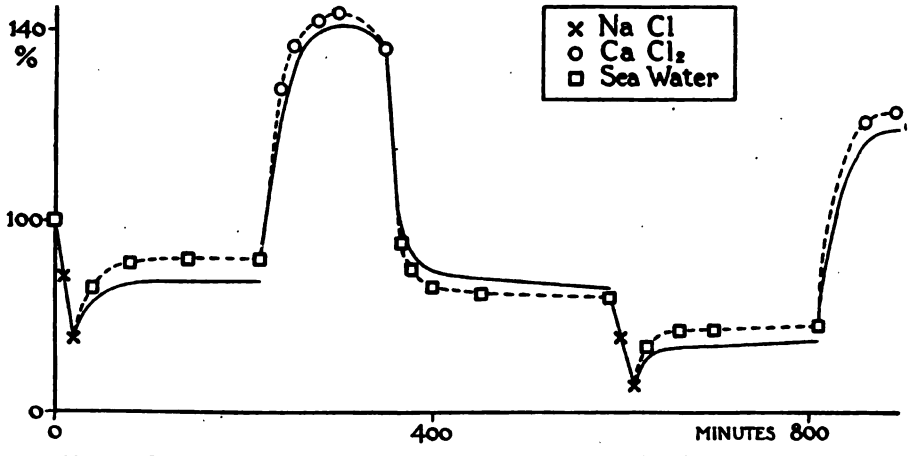

FIG. 3. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M, in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

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III. NaCl, Sea Water, $CaCl_2$, Sea Water, etc.

It seemed desirable to test the theory further by varying the experiments in the manner shown in Fig. 3. The calculations are made as already explained. It will be noticed that in this and in some other experiments the resistance rises rather more rapidly in $CaCl_2$ than the calculations would lead us to expect. This is due to the fact that the "standard curve" for $CaCl_2$, which was based upon previous experiments made under different conditions, seems to be a little too low for the present material.

IV. $CaCl_2$, NaCl, Sea Water, etc.

A series of experiments was made to determine the effect of $CaCl_2$ followed directly by NaCl. The results are shown in Fig. 4. The rise in $CaCl_2$ during the first 91.8 minutes is calculated in the usual manner. In order to calculate the subsequent drop in NaCl we must substitute in formula (7) the value of M; i.e., the observed resistance (less 10) at the beginning of exposure to NaCl. In place of 2313 we must substitute the value of A, which is $A_1 = 2700_e^{-(0.0018)91.8}$.

During the exposure of 60.6 minutes to NaCl the value of A changes from A_1 to $A_2 = A_1 e^{-(0.018)60.6}$.

This value must be substituted for A in Formula (1) in calculating the recovery in sea water.

In finding the value of S (by means of formula (3)) we must remember that during the 91.8 minutes in $CaCl_2$ the value of R (which at the start is $R_0 = 1041.77$) diminishes from R_0 to R_1 according to the formula

$$R_1 = R_0 e^{-91.8 K_R}$$

K_R in $CaCl_2$ = 0.012532 (See Table II of the preceding paper[8]). During the 60.6 minutes in NaCl R_1 diminishes to R_2 according to the formula

$$R_2 = R_1 e^{-60.6 K_R}$$

K_R in NaCl = 0.04998.

We must also bear in mind that O diminishes during the exposure. Since this process is 6 times as rapid in NaCl as in $CaCl_2$ we may

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consider 91.8 minutes in $CaCl_2$ to be equivalent to 91.8 ÷ 6 = 15.3 minutes in NaCl and the total exposure to be equivalent to 60.6 + 15.3 = 75.9 minutes in NaCl.[9] The value of *O* may then be found by means of formula (4).

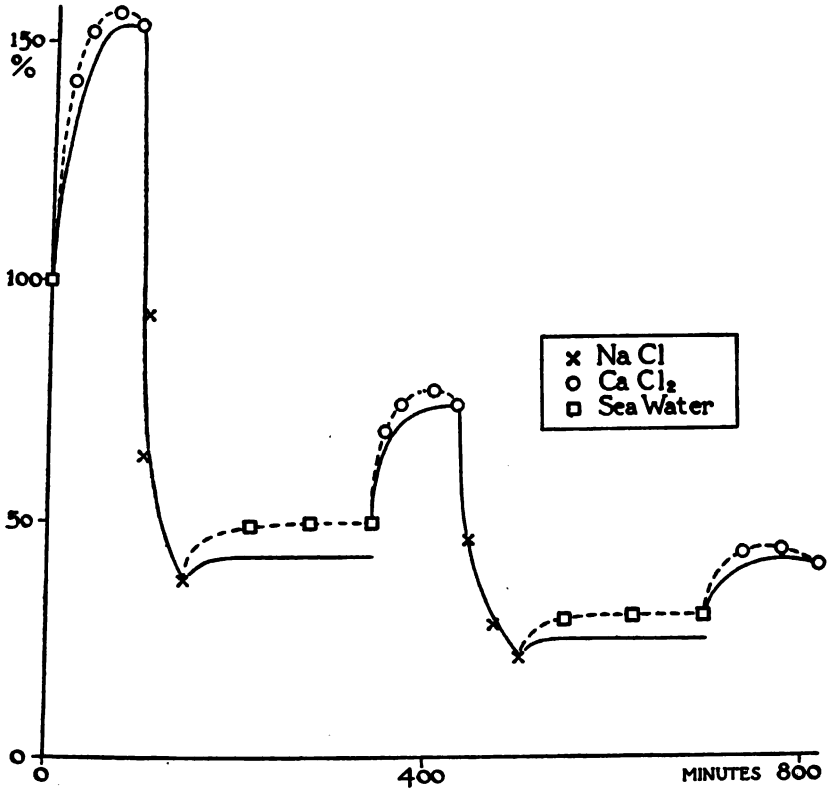

FIG. 4. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M, in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

[9] This involves the assumption that *O* is not restored to any extent during recovery in sea water. This assumption may not be quite correct, especially at the start, but even in that case the present calculation would not be appreciably altered.

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V. $CaCl_2$, NaCl, $CaCl_2$, NaCl, Sea Water, etc.

A series of experiments was performed in which tissue was placed in $CaCl_2$ for 30 minutes, then in NaCl for 10 minutes, then in $CaCl_2$ for 60 minutes. The tissue was allowed to recover in sea water, after which it was placed in $CaCl_2$ for 360 minutes, and then in NaCl.

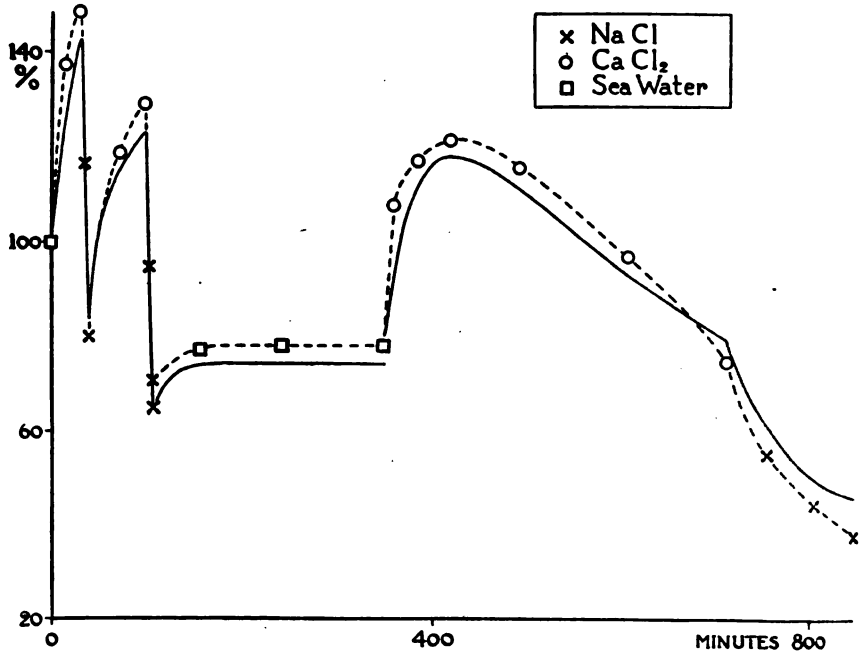

FIG. 5. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M, in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

In this case the observed time was not corrected (i.e., was not multiplied by a factor) as in the previous calculations. In consequence the calculated and observed values do not correspond at the beginning of each exposure, the only exception being after recovery in sea water, in which case it was assumed[10] that equilibrium had been

[10] In this case the tissue did not remain long enough in sea water to establish equilibrium but it was so nearly established that only a very small error is involved in regarding it as complete. In cases where it is not completely established the final equilibrium may be approximated with sufficient accuracy by extrapolation.

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reached and that in consequence $A_0 = 30M$ (the value of M being that of the observed resistance less 10). This value of A was taken for the subsequent calculations. During the subsequent exposure to $CaCl_2$ A_0 diminished to A_1 according to the formula

$$A_1 = A_0 e^{-(0.0018)360}$$

and this value was used in calculating the fall of resistance during the final exposure to NaCl.

Experiments similar to those shown in Figs. 1, 2, 3, 4, and 5 have been made, in which mixtures of NaCl + $CaCl_2$ have been used in a variety of ways. In this case we employ for the calculations the constants appropriate for each mixture, as given in a preceding paper.[8] In general the agreement is satisfactory.

It should be noted that we do not employ new constants to fit these curves but that in every case we use the constants already determined as the result of other and quite different experiments. In view of this the results have a special significance.

These experiments, and those described in previous papers, seem to afford a sufficient test of the theory. The agreement between the calculated and observed values appears to be satisfactory whenever a sufficiently large number of readings are averaged in arriving at the observed values.

It is evident that the equations which have been developed enable us to predict the behavior of the tissue under a great variety of conditions.

The mechanism which has been postulated in developing these equations consists essentially of a series of catenary reactions. There can be no doubt that catenary reactions play a large part in life phenomena and it would seem that the rôle assigned to them in the present discussion involves no unreasonable assumption.

It may be desirable to call attention to features of this mechanism which are of general interest from a theoretical viewpoint. It is evident that by means of a simple catenary system we can account for practically all the processes which occur in the organism. If such a system is present in the egg we can easily picture all of the subsequent development as due to this system, without the intro-

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duction of any new reactions. All that we need to postulate is that during development the relative rates of the reactions change. The processes involved in irritability, as well as those concerned in injury and death, may be accounted for in this way. We thus arrive at a very simple conception of the underlying mechanism of life processes, which may be useful in formulating a theory of living matter.

As an illustration of the effect of changes in relative rates we may take the substance, M, in the series

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B.$$

It is evident that we may increase the value of M in a variety of ways, as by increasing the rate of $O \rightarrow S$, $S \rightarrow A$, $A \rightarrow M$, or of any two of these reactions, or of all of them simultaneously. We may decrease the amount of M by decreasing the rates of these reactions or by increasing the rate of $M \rightarrow B$ (or increase M by decreasing this rate). We may likewise increase (or decrease) M by increasing (or decreasing) the amount of O. Furthermore if side reactions occur, such as $N \rightarrow O \rightarrow P$ or $R \rightarrow S \rightarrow T$ their rates will also affect the amount of M. It must also be remembered that any one of the substances in the system might act as an accelerator or inhibitor of any of the reactions. With such a system a great variety of processes is possible.

It is possible that such systems may play an important rôle in the fundamental processes of living matter.

SUMMARY.

Tissues of *Laminaria* transferred from sea water to solutions of pure salts, and thence to other solutions of pure salts, or to sea water, behave in a manner which can be predicted by means of the equations previously developed.

The behavior of the tissue may be explained as due to a series of catenary reactions. It is possible that a similar explanation may be applied to other fundamental life processes.

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THE RATE OF GROWTH OF THE DAIRY COW.

Extrauterine Growth in Weight.

By SAMUEL BRODY and ARTHUR C. RAGSDALE.

(From the Department of Dairy Husbandry, University of Missouri Agricultural Experiment Station, Columbia.)

(Received for publication, February 24, 1921.)

If a cell of the body of an animal is divided into two equal daughter cells, then clearly each daughter cell contains only half the chromatin contained in the mother cell. Before dividing again, however, the chromatin in each daughter cell is increased to the amount contained in the mother cell at the expense of the surrounding cytoplasm. This process of division and increase of chromatin is continued until a definite equilibrium ratio is reached between the cytoplasm and chromatin, when further division ceases, unless the cytoplasm continues to increase by further absorption of food. These facts, found by Sachs, Morgan, Driesch, Boveri, and others[1] and the further fact that the increase of chromatin at the expense of cytoplasm seems to be a chemical process, as seen for example by the dependence of the increase of chromatin on the presence of oxygen,[1] led Loeb[1,2] to advance the theory that the process of cell division is ultimately controlled by the fundamental chemical laws of mass action, and equilibrium. He also concluded from the fact that the cells multiply in a geometrical progression that the synthesis of chromatin is limited by an autocatalytic monomolecular reaction; that is, one which is accelerated by its own product.

What was said of the growth of chromatin must also hold true for the growth of the cell in the presence of food, and for the growth of any tissue and of the whole body of a metazoan; since the growth of the cell is in geometric progression, the growth of a tissue is the

[1] Cf. Loeb, J., The dynamics of living matter, New York, 1906, 58–66.

[2] Loeb, J., *Biochem. Z.*, 1906, i, 34; *Biol. Centr.*, 1910, xxx, 347.

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resultant of multiplication of cells, and the growth of the whole body is the resultant of the interdependent and harmonious growth of the various tissues. The whole body may therefore be said to be determined by a limiting autocatalytic reaction and as such the rate of growth of the mass of the body should follow the velocity equation of an autocatalytic monomolecular reaction. Robertson[3] and Ostwald[4] simultaneously but independently showed this to be the case.

The velocity equation of an autocatalytic monomolecular reaction may be derived as follows: Let A be the amount of limiting growth substance at the beginning of growth which is ultimately destined in the presence of nutrients to be converted into tissue; let the amount

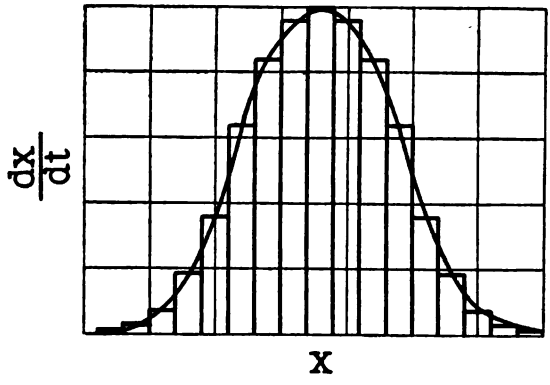

FIG. 1. Curve of $\frac{dx}{dt} = x(A - x)$. Ordinates represent velocity, $\frac{dx}{dt}$; abscissæ represent ascending values of x.

so converted be x; and, therefore, $(A - x)$ remains unconverted. By hypothesis, the velocity of growth $\frac{dx}{dt}$ is proportional to both $(A - x)$ and also to x which acts as the catalyzer of the reaction, that is,

$$\frac{dx}{dt} = Kx(A - x) \qquad (1)$$

[3] Robertson, T. B., *Arch. Entwicklungsmechn. Organ.*, 1907–08, xxv, 581; *Biol. Centr.*, 1910, xxx, 316; Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920.

[4] Ostwald, W., Vorträge und Aufsätze über Entwicklungsmechanik der Organismen, Leipsic, 1908, v, cited by Loeb (1910),[2] and Robertson (1910).[3]

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where K is the velocity constant. On plotting $\frac{dx}{dt}$ for various values of x a curve of a rising and falling type is obtained with a maximum at the center where $x = A - x$ or $x = \frac{1}{2}A$ as shown in Fig. 1.

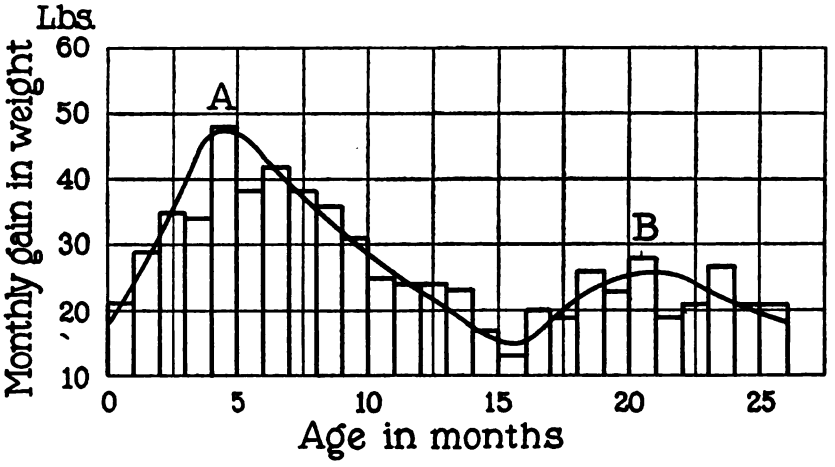

FIG. 2. Velocity curve of growth of the Jersey cow. Ordinates represent velocity of growth, the height of the rectangles representing monthly gains in weight; abscissæ represent age in months.

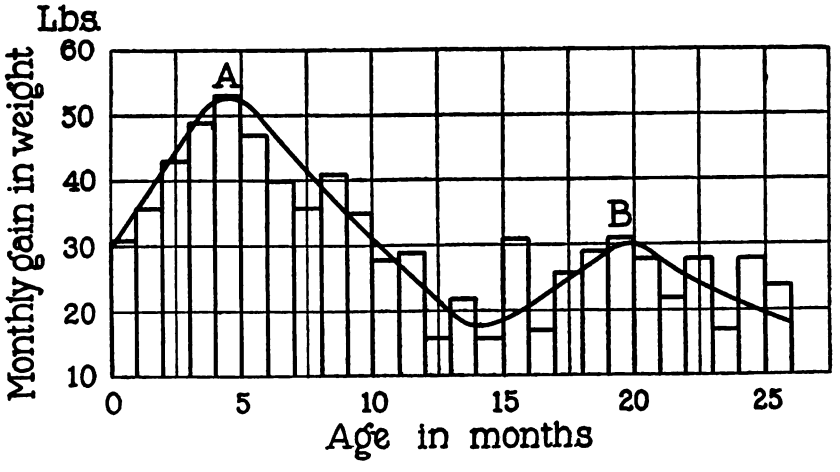

FIG. 3. Velocity curve of growth of the Holstein cow. Ordinates and abscissæ have the same meaning as in Fig. 2.

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A comparison of Fig. 1, the theoretical velocity curve of an autocatalytic monomolecular reaction, with Figs. 2 and 3, the velocity curves of growth of a group of dairy cows in this Station,[5] shows obvious similarities.

These postuterine velocity curves of growth show two reactions or cycles with maxima at about 5 and 20 months of age. The assymetry of the curve of cycle A may be tentatively explained by assuming that the second cycle, B, begins before the completion of cycle A, thus resulting in a superimposition of the two cycles with the consequent assymetry.

Other factors may conceivably enter, such as deposition of fat at that age, as suggested by Robertson in connection with his work.[3] The other irregularities might be expected considering that the animals were kept under average, which means highly variable, conditions and considering the small number of animals on which the data are based (Table I). The irregularities in the second extrauterine cycle B may be further accounted for by the fact that for reasons of economy the animals were bred at 20 to 21 months of age, calving at the ages of 29 to 30 months.

Cycle B is undoubtedly the last growth cycle, comparatively little growth being made after the age of 30 months. According to Donaldson[6] and Robertson,[3] there are at least three such cycles in the mammal; if the tricyclic theory is true, then there should be at least one cycle *in utero* in addition to the extrauterine cycles A and B. There is some evidence in this department, not enough, however, for publication, indicating that there is indeed such an intrauterine cycle.

Each of these cycles, according to Robertson,[3] should follow the equation of an autocatalytic reaction. It is interesting, in addition to the qualitative comparison of the experimental and theoretical curves, also to find out what is the approximate quantitative agreement between the experimental and calculated values. Equation (1) cannot be used for this purpose since, while the weights of the animals were recorded with reference to time, their age, the velocity

[5] Eckles, C. H., *Univ. Missouri Agric. Exp. Station, Research Bull. 36*, 1920.

[6] Donaldson, H. H., Boas memorial volume, New York, 1906, cited by Robertson (1907–08).[3]

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TABLE I.

*Data on the Growth of the Dairy Cow.**

	Holstein cows.					Jersey cows.				
Age.	Number of animals weighed.	Average weight.	Range in weight.†	Average deviation from average weight.‡	Deviation.§	Number of animals weighed.	Average weight.	Range in weight.	Average deviation from average weight.	Deviation.
mos.		*lbs.*	*lbs.*	*lbs.*	*per cent*		*lbs.*	*lbs.*	*lbs.*	*per cent*
0 birth	83	90	57			94	55	35		
1	9	121	23	4.3	3.6	6	76	31	10.7	14.0
2	9	157	22	5.6	3.5	7	105	34	11.0	10.5
3	9	200	48	13.1	6.6	7	140	54	14.4	10.3
4	9	249	66	15.3	6.2	8	174	73	18.5	10.6
5	9	302	97	20.2	6.7	9	222	83	22.0	9.9
6	9	349	129	21.9	6.3	10	260	101	23.7	9.1
7	11	389	169	34.0	8.8	10	302	107	20.8	6.9
8	11	425	217	31.5	7.4	10	340	134	22.7	6.7
9	11	466	251	41.8	8.95	11	376	152	23.5	6.3
10	11	501	268	50.5	10.0	11	407	146	23.6	5.8
11	11	529	291	35.7	6.7	11	432	134	35.2	8.0
12	11	558	276	39.9	7.2	11	456	124	35.9	7.9
13	11	574	237	36.3	6.3	11	480	118	34.3	7.1
14	11	596	243	38.6	6.4	11	503	127	25.8	5.1
15	12	612	186	42.9	7.0	11	520	114	31.8	6.1
16	13	643	164	42.2	6.6	11	533	87	27.4	5.1
17	14	660	174	45.7	6.9	12	553	95	29.3	5.3
18	14	686	162	36.0	5.3	13	572	78	22.4	3.9
19	14	715	183	33.0	4.6	13	598	126	31.4	5.2
20	14	746	123	31.9	4.3	13	621	130	32.6	5.2
21	14	774	216	47.0	6.0	13	649	151	32.6	5.0
22	14	796	223	51.5	6.5	13	668	108	33.3	5.0
23	14	824	177	43.1	5.2	12	689	179	47.6	6.9
24	14	841	151	38.8	4.6	12	716	133	40.2	5.6
25	14	869	188	48.6	5.6	12	737	147	39.3	5.3
26	14	893	234	42.0	4.7		758	188		
27	14	925	261	54.5	5.9		770	212		
28	14	966	253	55.0	5.7		784	92		
29	13	994	255	58.7	5.9		804			

* The animals were bred at 20 to 21 months of age, calving at 29 to 30 months of age.

† Range = difference between observed extremes; thus the observed extreme weights of Holstein calves were 55 and 112 pounds, a range of 57 pounds.

‡ Average deviation from average weight = $a.d. = \frac{\Sigma d}{n}$, a numerical measure of the amount by which a new observation taken under the given conditions is likely to differ from the average value.

§ Percentage deviation = $\frac{a.d.}{m} \times 100 = \frac{\text{Average deviation}}{\text{Average weight}} \times 100.$

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$\frac{dx}{dt}$ in equation (1) is not expressed as a function of time. Equation (1) is therefore first integrated. The integration is performed by writing

$$\frac{dx}{dt} = Kx(A - x)$$

in the form

$$dt = \frac{dx}{Kx(A - x)}$$

which is a standard form ready for integration

$$\int dt = \int \frac{dx}{Kx(A - x)}$$

Integrating and putting K for KA we get

$$t = \frac{1}{K} \log \frac{x}{A - x} + C$$

or

$$\log \frac{x}{A - x} = Kt + C$$

where C is the integration constant. The precise meaning of C, the integration constant, and t, time, may be obtained as follows: Let t, time, be counted from the maximum of the velocity curve (A in the figure); that is, where $x = A - x$. The value of t at the maximum point is therefore zero, so is $\log \frac{x}{A - x}$, and C also, and the equation is simplified to

$$\log \frac{x}{A - x} = Kt$$

where t is the time interval between the maximum point of growth and any other time on the curve. Instead of t, we may write $(t - t_1)$ when t_1 is the age of the animal at the maximum point of growth, and t is any age. The equation becomes

$$\log \frac{x}{A - x} = K(t - t_1) \qquad (2)$$

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where x, the weight of the animal, is expressed as a function of its age, t. Equation (2) is the form used by Robertson.[3] If desired, equation (2), a weight-age equation, may be changed to a velocity-age equation by differentiating it after some simplification and obtaining[7]

$$\frac{dx}{dt} = \frac{Ke^{Kt}}{(1+e^{Kt})^2}$$

where $\frac{dx}{dt}$ is the velocity, t and K have the same significance as in equation (2), $e = 2.718$, the base of natural logarithms. Equation (2) will, however, be used on account of the relative simplicity of its application to the data.

In equation (2), x is the weight gained from the beginning of any cycle up to any age, t, of that cycle; while A is the total growth made in that cycle which is equivalent to the limiting growth substance present at the beginning of the cycle. If the cycle under consideration is preceded by one or more other cycles, then the growth of the preceding cycles must be subtracted from the weight of the animal at the age t in order to obtain x. Let w be the weight gained up to the beginning of the cycle under consideration and x_1 the weight of the animal at the age t_1; then equation (2) becomes

$$\log \frac{x_1 - w}{A - (x_1 - w)} = K(t - t_1) \qquad (3)$$

The simplest method of applying equation (2) or (3) is first to plot the experimental data on growth in terms of monthly gains as was done in Figs. 2 and 3. The highest point of the velocity curve corresponds by definition to the age t_1. Having thus evaluated t_1, a reasonable beginning or end of the cycle is chosen by inspection; the growth made between the ages t_1 and the beginning or end of the cycle is the value of $\frac{1}{2}A$, since t_1 is in the center of the growth cycle. If the values of t_1 and $\frac{1}{2}A$ are properly chosen, the values of K for different values of x should be nearly constant. If the value of K deviates systematically then a slightly smaller or slightly larger value of $\frac{1}{2}A$ is chosen until the systematic deviations disappear; the

[7] *Cf.* Lewis, G. N., *Z. physikal. Chem.*, 1905, lii, 310; *Dept. Interior, Bureau Gov. Lab., Chem. Lab., No. 30*, 1905.

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value of A can be estimated still more easily by changing equation (2) $\log \frac{x}{A-x} = Kt$ to $y = Kt$ when y stands for $\log \frac{x}{A-x}$. Since $y = Kt$ represents a straight line therefore $\log \frac{x}{A-x}$, if plotted against t, should also give a straight line for the proper value of A. A few values of A are chosen and a few values of $\log \frac{x}{A-x}$ are plotted conveniently with the aid of semilogarithmic paper or still better by the aid of Robertson's tables.[8] The value of A giving the straightest line is the one chosen for computation. The last graphic method of verifying the correctness of the estimated value of A was called to our attention by Dr. E. R. Hedrick of this University, and it has proved very satisfactory in this work.

The first extrauterine cycle, A, should, in accordance with the tricyclic theory of growth, be preceded by another cycle *in utero*; that is, w in equation (3) should have some value other than zero. Since, however, we have no data on intrauterine growth of the dairy cow and since the neglect of the relatively small gain in the intrauterine cycle cannot affect the results very seriously, we shall for the present assume $w = 0$ and the weight gained up to t_1 is $\frac{1}{2}A$. Assuming the value of t_1 for the Holstein cow to be 4.5 months when the animal weighs 275 pounds (see Fig. 3), we get the value for A, $275 \times 2 = 550$ pounds, and equation (2) for the Holstein cow takes the form

$$\log \frac{x}{550-x} = 0.158\,(t - 4.5)$$

which should enable computation of comparable values of the animal to at least 9 months (2×4.5) of age which in fact is the case as shown in Table II.

After 9 months of age the observed values are greater for the probable reasons previously explained.

By the same method the equation for the Jersey cow is found to be

$$\log \frac{x}{444-x} = 0.169\,(t - 5)$$

[8] Robertson, T. B., *Univ. California Pub. Physiol.*, 1915, vi, 211.

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This equation gives an equally satisfactory agreement between the calculated and observed average values for the Jersey cow as seen in Table III.

TABLE II.

Age (t).	Weight observed (x).	Weight calculated (x).
mos.	*lbs.*	*lbs.*
0	90	90
1	121	120
2	157	157
3	200	202
4	249	250
5	302	301
6	349	348
7	389	393
8	425	430
9	466	461
10	501	485
11	529	503

TABLE III.

Age (t).	Weight observed (x).	Weight calculated (x).
mos.	*lbs.*	*lbs.*
0	55	55
1	76	76
2	105	105
3	140	140
4	174	180
5	222	222
6	260	265
7	302	305
8	340	340
9	376	367
10	407	389

The velocity curves of the last cycle, B (in Figs. 2 and 3) are very irregular, and no significance could be attached to the fit of equation (3) for the data of that period. The possible causes of their irregularity have been indicated. It might, however, be of interest to apply equation (3) to the data in order to illustrate the method of procedure. If the growth of the Jersey cow is taken as example it

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would seem reasonable from inspection of Fig. 2 to assume the value of t_1, the maximum, as 20.5 months of age when the animal weighs 635 pounds—the average weight of the Jersey cow at maturity having been found by Eckles[5] to be 902 pounds. A is therefore $2 \times 267 = 534$ pounds, and $w = 902 - 534 = 368$ pounds. Equation (3) after evaluation of $K t_1$ becomes

$$\log \frac{x_1 - 368}{534 - (x_1 - 368)} = 0.0765\,(t - 20.5)$$

Calculating x as before, we get Table IV.

TABLE IV.

Age (t).	Weight observed (x).	Weight calculated (x).
mos.	*lbs.*	*lbs.*
15	520	513
16	533	533
17	553	553
18	572	575
19	598	598
20	621	621
21	649	645
22	668	669
23	689	690
24	716	713
25	737	734
26	758	754
27	770	771
28	784	788
29	804	803

If all the assumptions are correct, and if pregnancy under the conditions at which the animals were kept did not interfere with their growth, then the observed weights before calving, at 29 months of age, should be greater by at least 55 pounds, the weight of the calf at birth, than the calculated weights. The effect of pregnancy on growth, however, is not well known, the evidence being somewhat contradictory,[9] and no conclusions can therefore be drawn from this rather too close agreement.

[9] *Cf.* Marshall, F. H. A., The physiology of reproduction, London, 1910. Minot, C. S., *J. Physiol.*, 1891, xii, 97. Watson, J. B., *J. Comp. Neurol. and*

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Table V shows the calculated and observed values during lactation. The large deviation of the calculated values from the observed values is in agreement with the established fact that lactation greatly retards growth.[9]

TABLE V.

Age (t).	Weight observed (x).	Weight calculated.
mos.	*lbs.*	*lbs.*
29	764	803
35	779	863
42	827	890
48	854	898
55	872	900
68	887	902
80	902	

SUMMARY.

The growth period of the Jersey and Holstein cows is made up of at least three cycles, two extrauterine cycles with maxima at about 5 and 20 months of age, and one intrauterine cycle, the maximum of which has not yet been determined. The equation of an autocatalytic monomolecular reaction was found to give very good results when applied to the cycle having its maximum at about 5 months of age. The values obtained from this equation when applied to the cycle having the maximum at about 20 months of age were higher than the observed values probably due to the retarding effect of pregnancy and lactation on growth.

We are indebted to Dr. Walter R. Bloor and Dr. Carl L. A. Schmidt, Department of Biochemistry, University of California, for valuable suggestions.

Psychol., 1905, xxv, 514. Eckles, C. H., *Univ. Missouri Agric. Exp. Station, Bull. 135*, 1915. Eckles, C. H., and Swett, W. W., *Univ. Missouri, Agric. Exp. Station Research Bull. 31*, 1918. Regan, W. M., The effect of lactation on growth, Thesis, Columbia, 1914. Griswold, D. J., A study of the effects of the periods of gestation and lactation upon growth and composition of swine, Thesis, Columbia, 1915.

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CASEIN VISCOSITY STUDIES.*

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INTRODUCTION.

Robertson[1] has shown that when casein is dissolved in dilute acids or alkalies (HCl or KOH), the viscosity of the solution increases with the increase in the concentration of the solvent. A similar set of results were obtained by Sackur.[2] That a maximum viscosity occurs in solutions of casein in alkalies has been pointed out by the writer upon another occasion.[3] Detailed publication of these results has been delayed because it was subsequently found that borax caused a displacement of this maximum viscosity[4] and it was very desirable to repeat with care the determination of the viscosity curves in order to be sure that no error in observation had been committed in the earlier experiments.

The viscosity-pH curves of casein in alkaline solutions only are considered in this paper. Viscosity-pH curves of pure casein in acids have been observed by the writer, but they are lacking in the practical significances manifested by the alkaline solutions. It may not be remarkable that all the commercial casein glues examined yielded mixtures, the pH values of which showed that the maximum viscosity had been widely overstepped, and that the viscosities of the mixtures lay on the alkaline flat part of the curve. The practical importance of knowing at what pH casein solutions show their maxi-

* Published by permission of the Secretary of Agriculture.

[1] Robertson, T. B., The physical chemistry of the proteins, Washington, 1918, 320–328.

[2] Sackur, O., *Z. physikal. Chem.*, 1902, xli, 672.

[3] Zoller, H. F., *Science*, 1919, l, 49.

[4] Zoller, H. F., *J. Ind. and Eng. Chem.*, 1920, xii, 1171.

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mum of internal friction outcrops in all industries where casein is used in the form of its solutions; *e.g.*, paper coating, sizings, paints, adhesives, nutritive preparations, dentrifrices, etc.

Two features are brought out in the viscosity curves which deserve separate treatment. On the one hand, as above mentioned, when casein is dissolved in sodium borate the maximum viscosity is attained at about pH 8.1 instead of pH 9.2 as is true for NaOH, KOH, and LiOH. Therefore a study of the anion effect is reported in Section II of these studies. Upon the other hand we observe a characteristic decline in the viscosity following the maximum and this decline is followed by a broad flattening of the curve. This effect has been correlated with the alkaline hydrolysis of the casein, whence numerous cleaved bodies are thrown into solution, such as ammonia, sulfides, phosphates, albumoses, etc. With the production of ammonia there is a flattening of the curve which has further significance when it is pointed out that solutions of casein in ammonia show a maximum viscosity, but this maximum, once attained at pH 9.2, continues without the characteristic decline. Thus, it will be appreciated that solutions of casein in ammonia generally maintain a higher viscosity. In Section III, therefore, the effect of alkaline hydrolysis upon the viscosity of casein will be dealt with separately.

Section IV will treat very briefly of the influence of high temperatures upon the viscousness of casein solutions, inasmuch as the author has treated of this factor somewhat more in detail in connection with the revision of the borax solubility test for caseins.[4]

I. Relation Between pH and Viscosity of Solutions of Casein in Alkalies.

Since the earlier investigators in dealing with casein solutions made no attempt to study the effect of hydrogen ion concentration upon their viscosity it was found expedient to redetermine the viscosity curves of casein in alkalies using the hydrogen electrode as a check upon the reaction of the solutions. Chick and Martin[5] measured the effect of concentration of casein upon the viscosity but did not indicate whether or not the reaction of the solutions was held at constant pH. Consequently the writer has repeated these measurements,

[5] Chick, H., and Martin, C. J., *Kolloid. Z.*, 1912, xi, 102.

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being certain that the reaction of the casein solution was the same in all concentrations. pH 9.0 was chosen for these measurements because at this point we are in the region of maximum viscosity of casein in the ordinary alkalies.

Preparation of Solutions.—Alkalies.—Sodium, potassium, and lithium hydroxides were freed as far as possible from their carbonates by preparing concentrated solutions and allowing the carbonates to crystallize, then filtering or pouring the clear solutions from the precipitate. Molar solutions were then prepared of the respective compounds; M/1 solution of freshly distilled NH_3 was also prepared.

Casein.—Casein was obtained by purifying a quantity of imported casein according to the method of Hammarsten as modified by Robertson.[1] This was designated by the symbol *R*. A casein, the history of which was completely known, so as to overcome the question of heated casein, was prepared by precipitating the casein from fresh, unpasteurized skim milk with dilute HCl according to the grain curd method.[6] The casein was then dissolved in ammonia and reprecipitated twice in order to free it from salts and other impurities. It was then dried with concentrated alcohol without the use of heat. This casein was designated as *G* in the following studies.

In the preparation of all casein solutions for the different studies the casein was carefully weighed into a dry flask and 50 cc. of distilled water, which had been freshly boiled and cooled by drawing CO_2-free air through it while immersed in running cold water, were added with thorough shaking to prevent the finely divided casein from forming lumps. After soaking in the water for 30 minutes the requisite quantity of alkali (M/1) was added from a burette. The mixtures were set in a water bath controlled at 30°C. and when all the casein had dissolved (2 hours were allowed) they were made up to final volume with the distilled water described above.

Measurement of Viscosity.—Although many of the measurements of viscosity of casein solutions have been conducted in the past with the usual type of capillary viscosimeter the present work was done almost entirely with a rotating disc instrument. The convenience of this latter type of instrument for the heavy casein solu-

[6] Clark, W. M., Zoller, H. F., et al., *J. Ind. and Eng. Chem.*, 1920, xii, 1163.

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tions is at once appreciated and for comparative measurements of such protein solutions is probably more nearly representative of their true internal friction because the measurements can be made quickly and with little molestation of their physical structure.[7]

The writer chose the MacMichael viscosimeter which embraces the rotating disc because of its availability to those interested in checking the viscosity values of casein solutions. The double brass bowl furnished with the instrument was replaced by one designed especially for these investigations. The essential difference was the provision for accurately controlling the temperature of the water bath in the outer chamber so that the viscosity measurements could be made at known temperatures. Fig. 1 is a cross-sectional diagram of the bowl used. A represents the removable inner bowl of brass for holding the solution under measurement; B, the outer bowl which is made to fit on the turntable of the instrument; C, the brass supports for the inner bowl; D, a small spiral of nichrome wire wound around a silica tube for the heating unit, and is inclosed in a small tube of pyrex glass; E, a small mercury thermoregulator. D and E are suspended in the water bath F and held rigid; the rotation of the bowl by the driving mechanism produces the stirring effect. D and E are further operated through a relay with carbon lamps in series in the circuits. G is the suspended disc furnished with the instrument. (For further comments on the MacMichael viscosimeter see Herschel.[8])

The values given for the viscosity of the various casein solutions are those obtained by subtracting the viscosity of the solvent at the temperatures measured from the total viscosity of the solution expressed in the divisions marked on the dial of the instrument. They are not absolute values, therefore, but only relative; the same relation would doubtless hold for any type of viscosimeter. The viscosity was measured at 25°C.

Determination of pH.—In the first instances of the work the reaction of the casein solutions was gauged by the use of the sulfonephthalein indicators similar to the method since described by Haas.[9] Five drops of the casein solution were placed on the inner surface of

[7] Garret, H., *Phil. Mag.*, 1903, vi, 374.

[8] Herschel, W. H., *J. Ind. and Eng. Chem.*, 1920, xii, 282.

[9] Haas, A. R. C., *J. Biol. Chem.*, 1919, xxxviii, 49.

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a small porcelain crucible lid and one drop of a water solution of the indicator was added (0.04 per cent indicator solution). This was compared to the same quantity of indicator in a mixture of four drops of buffer standards (Clark and Lubs standards) plus one drop of 1 per cent starch paste, on a similar background.

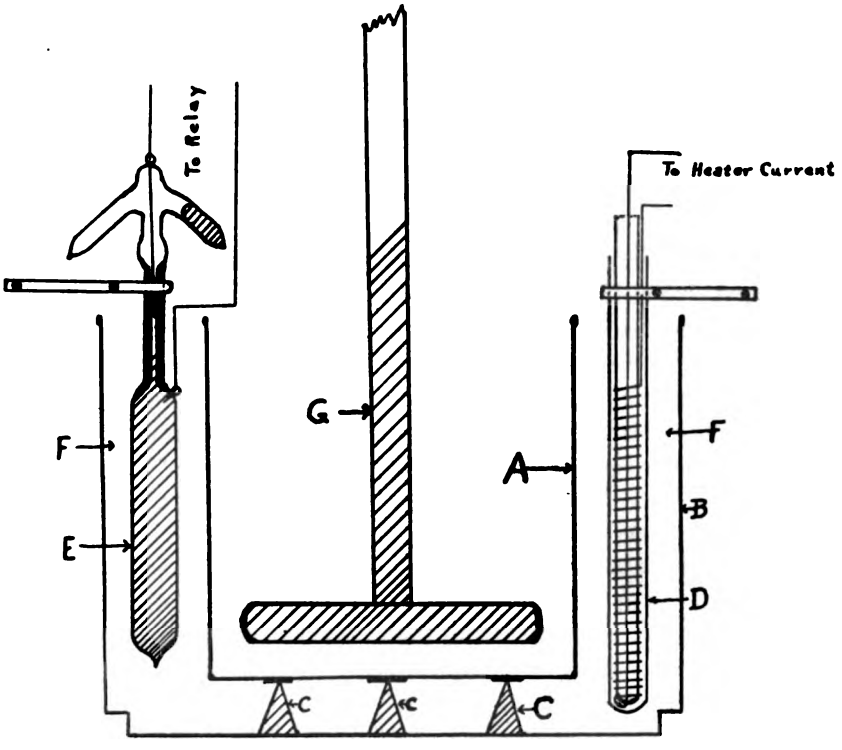

FIG. 1. Viscosimeter bowl.

It was later found that the values given by the above method were incorrect and the error was not constant but varied with the indicator and with the region of the pH scale. The hydrogen electrode was used, it consisted of the rocking electrode type described by Clark,[10] using saturated KCl junction and saturated calomel electrode. The platinum electrodes were first plated with gold and then with

[10] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

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a smooth coat of iridium. A well plated electrode lasted during one complete run of a single series of casein solutions titrating from the acid end of the series toward the alkaline end, usually about eight consecutive determinations.

The hydrogen ion concentrations of the various solutions were determined without dilution at 30°C. They were also determined after equal time intervals of contact of casein and solvent (2½ hours), immediately following the viscosity determination.

Effect of the Concentration of Caseinate on the Viscosity at Constant pH.

As previously mentioned pH 9.0 was chosen as the arbitrary reaction to which all the solutions were adjusted for these measurements. Casein *R* was employed and it was measured into wide mouthed flasks in the quantities necessary to yield the various concentrations represented on the curve in Fig. 2 as the experimental points. They were soaked in water, and the required amount of M/1 NaOH was then added to reach pH 9.0 (electrometric) after adjusting to final volume. In calculating the concentration of caseinate, allowance was made for the moisture (2.10 per cent) in casein *R*. Fig. 2 shows graphically the nature of the concentration effect. It confirms Chick and Martin's results.[5]

Viscosity-pH Measurements of Casein in NaOH, NH_4OH, KOH, and LiOH.

The interest in the foregoing viscosity-concentration curve lies in choosing the proper concentration of caseinate solutions for the extensive viscosity measurements. By reference to Fig. 2 we find that a 9 to 10 per cent caseinate solution is in the region of maximum sensibility to differences in reaction and to other factors such as salts, or ion effects. It was decided, therefore, to dissolve 10 gm. of Casein *R* or *G* in 100 cc. of solvent solution in every case. As the alkali was varied in each mixture the water was varied correspondingly so that the total volumes of the two were always 100 cc. This gave solutions containing approximately 9 per cent of caseinate.

For brevity the tables of each individual set of experiments are omitted and the data as far as possible are presented in Fig. 3. Only

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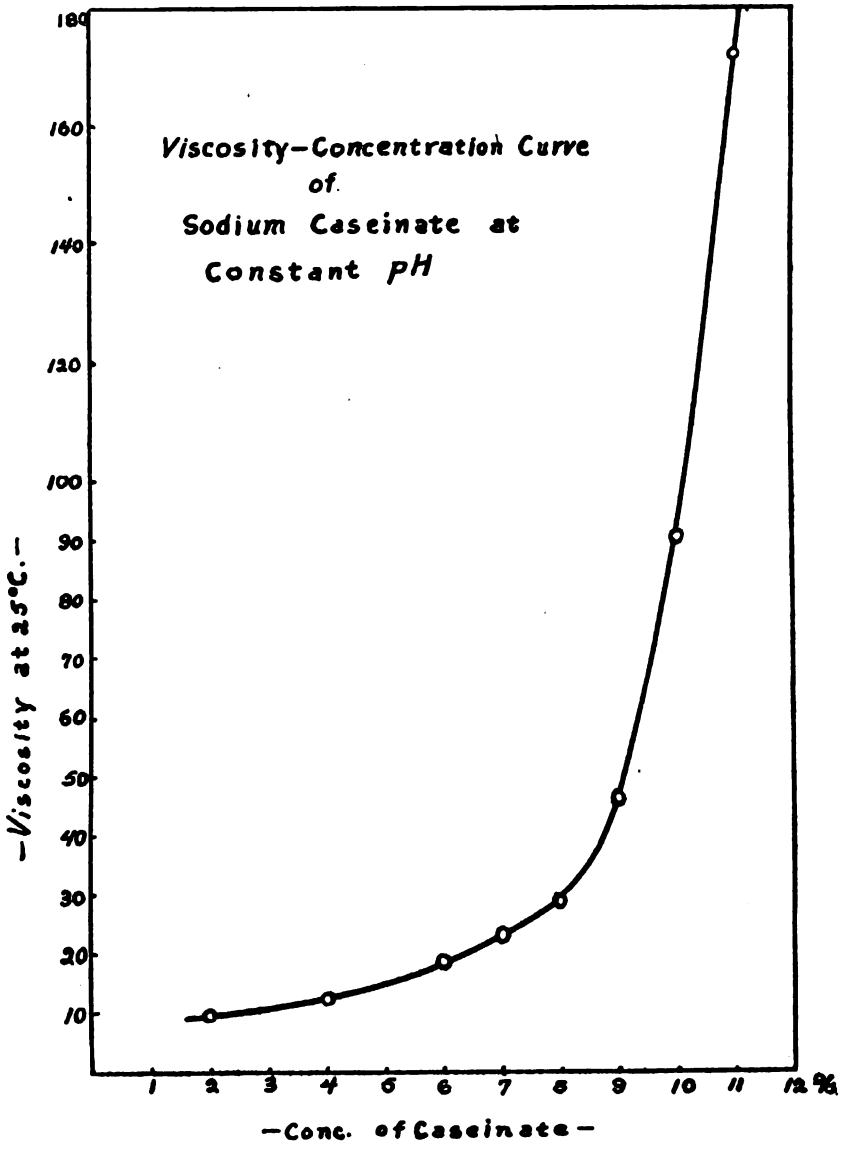

FIG. 2. Viscosity-concentration curve.

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the data for *G* dissolved in NaOH are included, since *G* seemed to conduct itself in all respects similar to *R*. All the pH values are the result of electrometric determination. Fig. 3 contains the viscosity-pH curves of the four hydroxides studied.

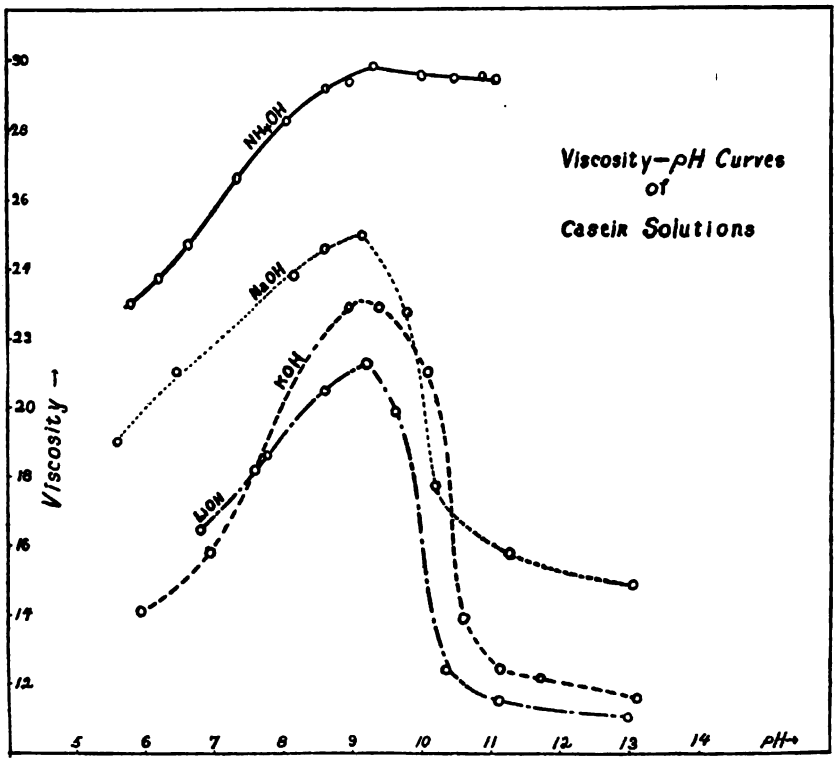

FIG. 3. Viscosity-pH curves of casein dissolved in the respective alkalies.

There are two noticeable characteristics among the various curves. In the proximity of pH 9.0 the different alkalies reach their maximum viscosity with casein. This is so uniform that we are safe in saying that casein shows its maximum viscosity at this reaction except, as the data in Section II will show, when it is dissolved in the presence of certain anions such as borates. The magnitude of this maximum viscosity varies somewhat with the alkali, being greatest with NH_4OH and least with LiOH, though the difference is apparently of little immediate importance.

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The viscosity drops precipitously following the maximum with each alkali except NH_4OH. In this case there is a slight fall but it continues to remain high even in great concentrations of ammonia (50 cc. of M/1 to 10 gm. of casein). At pH 10.5 with NaOH, KOH, and LiOH there is an evolution of NH_3 from the solutions, shown by white fumes with concentrated HCl and by turning methyl red to yellow (methyl red paper was held above the solution). Following pH 10.5 the viscosity is constant and about parallel to the ammonium hydroxide curve. This serves to show that in these concentrations of alkali the casein is furnishing NH_3 to the solutions so that they simulate the influence of the pure ammonium curves. Hence the flattening could be attributed to the accumulation of NH_3. This will be dealt with further in Section III.

Robertson calls attention to the relation between the ionization of protein solutions and their viscosity.[1] The viscosity increases with the electrolytic dissociation of the proteins; of course as ionization is conceived to be zero (or in electrochemical equilibrium) at the isoelectric point, we find simultaneously with the isoelectric condition a minimum viscosity. Hence Robertson points out[1] that at the point of maximum base-binding capacity, or saturation with base, there should be a maximum viscosity. If we note the quantity of NaOH that is necessary to cause the casein to attain its maximum viscosity at pH 9.2 we find that for each gram of moisture-free casein 0.98 cc. of normal NaOH was required (average of eight determinations). At this point 1 gm. of casein had combined with 98×10^{-5} gram equivalents of alkali. This value is slightly greater than one-half the value obtained by Robertson (180×10^{-5}) using the gas chain method.[11] The writer employed very concentrated solutions (8 to 10 per cent) of casein, while Robertson worked with very dilute solutions and this may be responsible for the difference in observations. In two instances, the writer allowed the concentrated casein solutions

[11] Robertson, T. B., *J. Physical Chem.*, 1910, xiv, 528.

The high concentrations of casein used by the writer have given values for the pH of the maximum viscosity, considerably different from those observed by Loeb in working with dilute solutions. (Loeb, J., *J. Gen. Physiol.*, 1921, iii, 357.) The interval between the preparation of the casein solution and the determinations of the pH seemed ample for the equilibrium.

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to stand for 30 hours, but the relative values remained the same; that is, the maximum viscosity occurred when the casein had combined with $90-100 \times 10^{-5}$ gram equivalents of base. When 180×10^{-5} gram equivalents of alkali were added to 1 gm. of casein in the writer's studies, the viscosity had fallen to a minimum, or on the flat part of the curve, and the pH was greater than 10.5. This would indicate that hydrolysis is taking place at this combining capacity, or at least it is in the first stages.

II. Anion Influence on the Viscosity of Casein Solutions.

The anions studied in this connection were those resulting from the ionization of the following salts: Na_3PO_4, NaF, Na_2SO_3, $Na_2B_4O_7$, Na_2CO_3, Na_3AsO_4, and Na_2SiO_3. Casein is readily soluble in solutions of the above salts. It is also readily soluble in solutions of the neutralized acids (neutral sodium salts), such as citric, oxalic, tartaric, etc., but these viscosity data are not presented.

The method used in preparing the casein solutions was essentially the same as that for the pure alkalies described in Section I. The casein was first soaked in 50 cc. of water and the solvent then measured into each quantity; they were then made up to volume. After the regular time interval ($2\frac{1}{2}$ hours), the viscosity and hydrogen ion concentration were measured exactly as in Section I. Fig. 4 shows the viscosity curves plotted from the experimental data.

The curve for sodium carbonate is necessarily similar to the NaOH curve. Naturally, if the curves represented the data for viscosity plotted against concentration of salt solution instead of pH we would observe wide variations in the gradient of the various curves corresponding to the differences in the dissociation of the solvents. Since the degree of internal friction varies more pronouncedly with the pH than with the concentration of molecules of the solvent, we are correct in representing the values as given in Fig. 4. It was impossible to use the hydrogen electrode in the sodium sulfite solutions for the determination of pH, because of fluctuating potentials probably resulting from sulfur dioxide which permeates the solutions. The dissociation constant of sulfurous acid is less than that of casein and this accounts for the formation of SO_2 in the solutions. The pH was determined roughly by the spot test with sulfonephthalein indicators.

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The curve for sulfite is not presented because of the inaccurate pH data. But a maximum viscosity was obtained, using the same time interval as employed in the other data, in a region corresponding to a pH of 6.4 to 6.6. What this may mean is difficult to interpret with the meager data at hand. No attempt was made to remove SO_2 from the solutions before taking measurements.

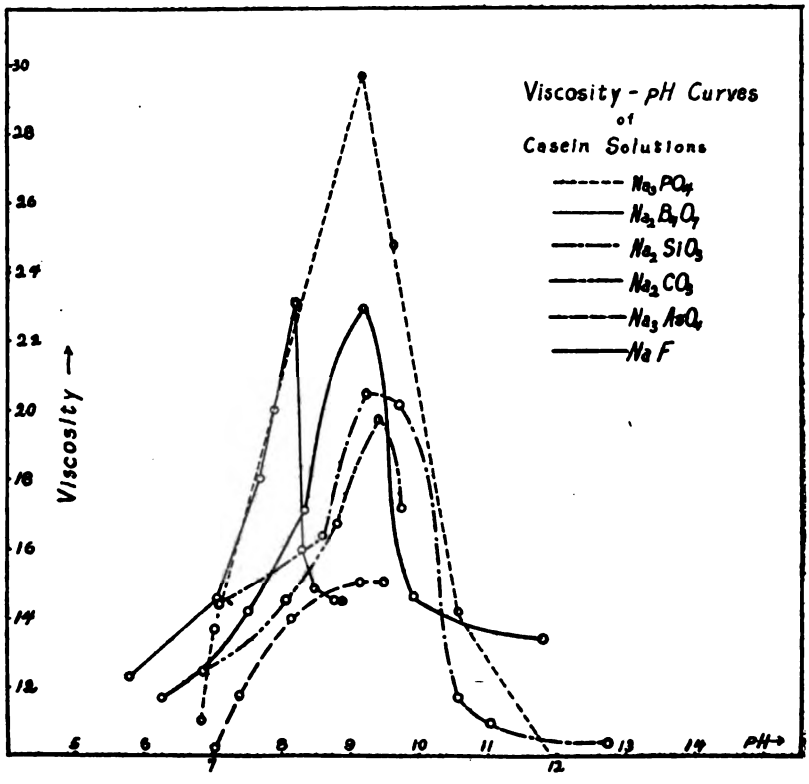

FIG. 4. Viscosity-pH curves of casein dissolved in the various solvents. Anion effect on the viscosity maximum.

With sodium arsenate a precipitation was observed at the point of maximum viscosity; the precipitate increasing in quantity with the increasing pH. The hydrogen electrode potentials were very steady. On the other hand sodium silicate solutions of casein gave unsteady potentials, although they were somewhat more steady than with sulfite. The pH values for sodium silicates as given are correct within 0.1 pH.

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Many of these salts are employed in casein water-resisting glues to combine with the excess of calcium oxide used as solvent for the casein. Certain ones will effect the setting time of the glues, thereby regulating the smoothness of the product. The above data on viscosity influence of these salts should indicate the choice of correct solvents for particular purposes.

The peculiar behavior and place of borax among these curves should not be passed without remarking that the phenomenon may possibly be traced to the influence of polyhydroxyl groups upon the dissociation of boric acid. Glycerol, mannitol, and various sugars are known to cause increased dissociation of boric acid which permits of the determination of boric acid by titration. The presence of tyrosine in casein suggests that casein may contain free hydroxyl groups, and these have indeed been demonstrated by the preparation of acylated products. It is possible that casein will act similarly toward boric acid thus effecting the displacement of the maximum viscosity.

III. Alkaline Hydrolysis of Casein as Influencing Its Viscosity.

Section I of this series showed that when casein is dissolved in alkalies other than NH_4OH the viscosity of the solutions containing the same concentration of casein increases as the pH increases up to 9.2 after which the viscosity sharply drops to the region of pH 10.5 after the viscosity remains fairly constant. In case of NH_3 solutions of casein there is not such a drop after reaching the maximum viscosity (temperature of solutions 25°C). The odor of all casein solutions, the pH of which are pH 10.0 or more, is distinctly ammoniacal. Moist litmus paper becomes blue when held above these solutions, while strong HCl evolves dense white fumes when brought in their vicinity.

Dakin and Dudley[12] have prepared what they term racemized casein by treating casein with 2 per cent NaOH for several hours, or until the specific rotation has decreased to zero. They observe that ammonia is evolved, that the racemized casein contains a low content of phosphorus, and describes the process of decrease in rotation

[12] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 263.

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as one of racemization or a keto-enolic tautomerism. Whatever explanation might serve to describe the change taking place in the molecule to effect the optical properties, there is a far greater fundamental change taking place in the casein molecule in the presence of these concentrations of alkalies which has received little attention. Maynard[13] has shown that NaOH of 2 per cent concentration also cleaves loosely combined sulfur from the casein molecule, a fact apparently noted by Dakin and Dudley.[12]

The writer has been able to prepare an acid-precipitable product, from the action of 2 per cent NaOH upon casein, that contains no sulfur, no nitrogen in the free amino form, and only a trace of phosphorus. The action of the alkali upon the casein proceeded for 6 days at 30°C. The gelatinous solution was diluted with water and treated with dilute acetic acid. The curd or precipitate was collected and thoroughly washed with water containing a trace of acetic acid. It was then thoroughly washed with distilled water and dried to constant weight, and analyzed for amino nitrogen, sulfur, and phosphorus. The action of alkali upon the casein has been more severe than a racemization or enolization, and it is quite likely that the loss of amino nitrogen, sulfur, and phosphorus from the casein molecule is partially responsible for the decline in rotation observed by Dakin and his coworkers.

In applying this knowledge to the explanation of the flattening of the viscosity curves in the alkaline zone the writer has found that the cleavage of sulfur and phosphorus is simultaneous with the cleavage of amino nitrogen. They both commence at nearly the same intensity of hydroxyl ion concentration, or, reciprocally, at about the same pH (10.0 to 10.5). The hydrolysis also commences as soon as the casein is dissolved in solvent at this pH. Within 6 hours nearly ½ the phosphorus and sulfur had been cleaved from the casein at pH 11.1 (9 per cent casein solution). When a few drops of $CuSO_4$ solution are added to the flasks containing solutions of casein for viscosity measurements, it is observed that the characteristic biuret color develops within a few minutes in those solutions which are found to evolve ammonia, and the depth of violet color increases with time and

[13] Maynard, L. A., *J. Physical Chem.*, 1919, xxiii, 145.

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with increasing pH. All casein solutions at pH 10.0 to 10.5 and higher pH responded to the copper reaction; if dilute acid is added to the casein solutions, which contain copper, and give the violet color, a dark color is formed owing to the formation of peptized copper sulfide.

The protein body which is precipitated from the alkaline hydrolysate of casein by the addition of dilute acids is, as described by both Dakin and Maynard, very gelatinous. It forms a clear transparent gel of high viscosity. The formation of this hydrolytic product from casein in the presence of the liberated NH_3 is believed to be responsible for the flattening of the viscosity curve at pH greater than 10.0.

The quantity of nitrogen split off from casein by the alkaline hydrolysis was found to be nearly $\frac{1}{2}$ the lysine nitrogen of the original casein and designated as free amino nitrogen by Van Slyke and Birchard.[14]

It can be stated with a sense of security that in solutions originally containing casein of which the pH are greater than 10.0, we are no longer dealing with casein but with protein-cleaved products. The flattening of the viscosity curve is coincident with the alkaline hydrolysis of the casein.

Furthermore the glues and adhesives formed from casein are ultimately a mixture of these cleaved products. It seems necessary now to determine the relative adhesive power of pure caseinate and the protein body which constitutes the larger bulk of the casein alkaline hydrolysate.

IV. Temperature as Effecting the Viscosity of Casein Solutions.

If we heat solutions of casein in alkalies (NaOH) to the boiling point and allow the solutions to cool spontaneously the resulting viscosities as measured at 25°C. are very little different from the viscosities of the unheated solutions at equivalent pH. Upon the other hand if the casein is precipitated from its solutions, after they have been heated, by acid and is then dried the resulting casein again dissolves in alkalies to furnish solutions of much higher viscosity.

[14] Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913, xvi, 539.

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Upon another occasion the writer has shown the effect of the heat treatment of milk upon the optimum temperature for acid precipitation of casein[15] and at the same time presented some data to show that this denaturation of the casein affected its hydrophyllic properties.[6,15] Again the conduct of casein, heated and unheated, toward borax solutions in the borax solubility test[4] emphasized that casein which had been subjected to high temperatures imbibes more water in solution and thereby produces greater internal friction or viscosity; in many instances they gel where normal casein solutions flow readily. The writer has further shown the formation of β casein in highly heated casein solutions.[16]

To show the effect of heat treatment of casein upon its viscosity in NaOH several experiments were conducted, two of which are described. Fresh skim milk was divided into two portions, *A* and *B*. The casein was precipitated from *A* according to the normal grain curd method[6] without heating the milk externally above 34°C. *B* was pasteurized at 65°C. for 45 minutes and the casein was precipitated from it with the modified grain curd method described by the writer.[14] The two moist caseins were dried under the same conditions in an air current at 40°C. The viscosities of the resulting caseins A_e and B_e were measured at 25°C. in the MacMichael apparatus as modified. Casein B_e contained 0.45 per cent more ash than A_e.

EXPERIMENT I.

Casein.	Viscosity.	pH	Concentration of casein.
			per cent
A_e	39.2	9.15	8.8
B_e	49.3	9.12	8.7

The other experiment mentioned above consisted in pasteurizing a 6.0 per cent solution of Casein *R* dissolved in NaOH at 80°C. for 30 minutes. The solution was cooled thoroughly and its viscosity at 25°C. was compared to the viscosity of a similar solution that had not been pasteurized.

[15] Zoller, H. F., *J. Ind. and Eng. Chem.*, 1921, in press.
[16] Zoller, H. F., *Chem. Abst.*, 1921, xv, 401–02.

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EXPERIMENT II.

Casein.	Viscosity.	pH
Pasteurized solution	25.4	6.43
Unpasteurized solution	27.6	7.16

These two experiments are typical of what we can expect of the effect of heat upon casein as affecting its viscosity. The results at

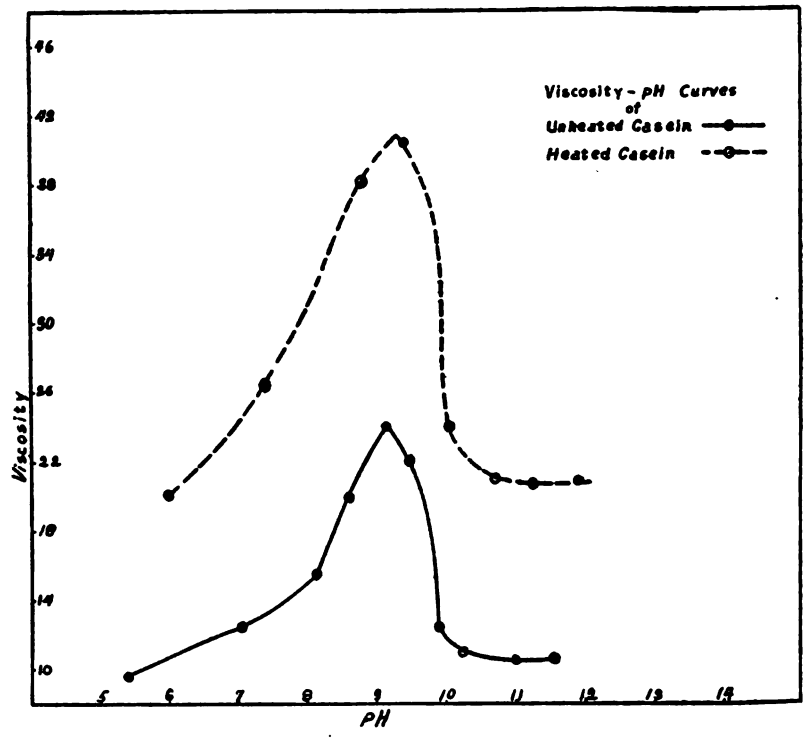

FIG. 5. Viscosity-pH curves of heated and unheated caseins.

first seem to be contradictory. But in the light of viscosity data obtained with milk the results of Experiment 2 show that the decreased viscosity by pasteurizing can be entirely accounted for through the decreased pH of the solutions, provided that we ignore the effect of heat on the coaguable proteins and casein. In milk, pasteurization generally causes a drop in pH of 0.10 to 0.20 and a drop in viscosity

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with the MacMichael instrument from 71.0 before pasteurizing to 69.0 after pasteurization. When the casein is separated from the milk serum salts and dried, its hydrophyllic properties are changed and it can then imbibe more water. Inasmuch as borate solutions buffer in the region of maximum viscosity of casein solutions it is peculiarly well suited to the differentiation of heated and unheated caseins, from the standpoint of their power of imbibition.

Fig. 5 shows the viscosity-pH curves of two caseins; one of a casein prepared without heat treatment, the other of some of the same casein precipitated from NaOH solution (pH 7.2) after the solution had been boiled for 20 minutes; the casein was dried with alcohol. The viscosity of the heated casein is nearly twice that of the unheated. The conditions of measurements were the same and the solvent was M/1 NaOH.

SUMMARY.

1. Viscosity and pH curves of casein dissolved in NaOH, KOH, LiOH, and NH_4OH are shown and it is found that a maximum viscosity occurs at about the same pH point with each alkali; *i.e.*, 9.1 to 9.25. The magnitude of the viscosity is largest in ammonia solutions.

2. The maximum viscosity occurs in 8 to 10 per cent solutions of casein in alkalies when about 98×10^{-5} gram equivalents of base are combined with 1 gram of casein.

3. A maximum viscosity occurs in the same region (pH 9.1 to 9.25) when casein is dissolved in Na_2CO_3, Na_3AsO_4, Na_2SO_3, NaF, and Na_2PO_3.

4. The maximum viscosity obtained with borax solutions of casein occurs at 8.15 to 8.2 pH. It is suggested that casein acts like mannitol, glycerol, etc., in increasing the dissociation of boric acid.

5. The flattening of the viscosity curves of casein solutions, following the decline from maximum, is shown to be due to alkaline hydrolysis whence casein no longer exists as such but is cleaved into a major protein containing no phosphorus or sulfur and less nitrogen. This cleavage commences at pH 10.0 to 10.5.

6. When casein is prepared from solutions that have been subjected to high temperatures (60°C. and above) or has otherwise been heated during its preparation, it yields solutions in alkalies of high viscosity.

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THE SIGNIFICANCE OF LATENCY TIME IN ENZYME DETERMINATION.

By LOURENS G. M. BAAS-BECKING.

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(Received for publication, January 18, 1921.)

Bredig and von Berneck,[1] in their work on inorganic ferments, determined the catalytic activity of platinum and manganese salts on hydrogen peroxide by means of titration with potassium permanganate. In many of their experiments they found, however, that the reaction velocity increased during the reaction. Bredig and Marck[2] attempt to explain this fact by a gradual activation of the catalyzer by the substrate. Denham[3] believes that the increase in reaction velocity is due to absorption at the surface layer. The results of Waentig and Steche[4] on blood catalase seem to indicate that the increase in reaction velocity is not a constant feature of the catalase; they found a constant, or even a decreasing, reaction velocity. Senter,[5] however, obtained opposite results with the same enzyme; there were cases in which the velocity increased. It seems to me that the controversy between different authors can, at least partially, be solved by the following considerations.

As I was able to demonstrate, the reaction does not start immediately after mixing the hydrogen peroxide with the enzyme solution. For this experiment I used an autographic registration of catalase action in a manometer. A certain latency period shows itself before the reaction starts. Probably an adsorption compound is formed during this latency period, which on decomposition liberates the oxygen. In one case, I obtained a latency time of 98 seconds while

[1] Bredig, G., and von Berneck, R. M., *Z. physikal. Chem.*, 1899, xxxi, 258.

[2] Bredig, G., and Marck, A., Gedenkboek aangeboden aan J. M. Van Bemmelen, 1910, 342.

[3] Denham, G. H., *Z. physikal. Chem.*, 1910, lxxii, 686.

[4] Waentig, P., and Steche, O., *Z. physiol. Chem.*, 1911, lxxii, 256.

[5] Senter, G., *Z. physikal. Chem.*, 1903, xliv, 257.

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the reaction was completed in 2,058 seconds (Fig. 1). The abscissa represents the time; the ordinate the amount of substance decomposed under the influence of the enzyme. Let A' be the time when the enzyme is added, A the time when the reaction starts, and A'-A the latency time T°; $A' - P = T'$; $A' - Q = T''$. If the reaction is monomolecular, the equation of the line ABC will be

$$K = \frac{1}{T} ln \frac{a}{a - x}$$

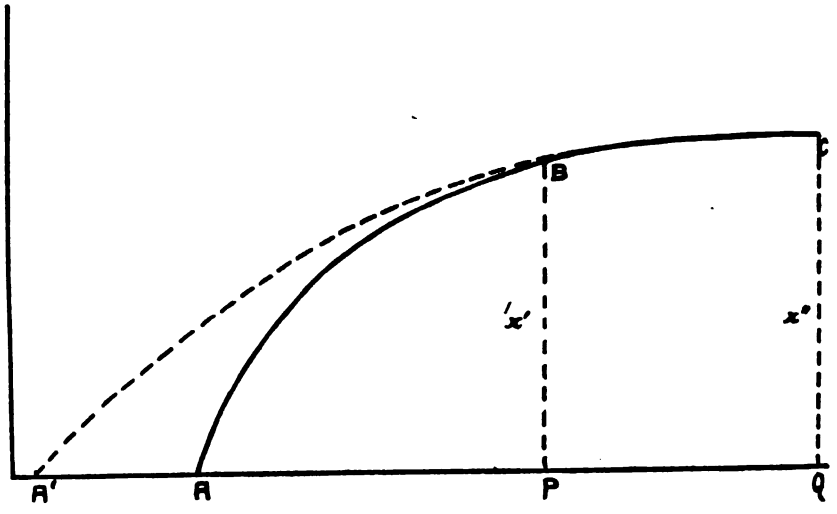

FIG. 1.

in which K represents the reaction velocity, T the time, a the available amount of substrate, and x the amount decomposed. If the reaction is supposed to be polymolecular the following formula will apply.

$$K = \frac{a^n - (a - x)^n}{T . a^n (a - x)^n}$$

in which n-1 is the order of the reaction.

The proportion of T' and T'' will be

$$\frac{T'}{T''} = \frac{ln \frac{a}{a - x'}}{ln \frac{a}{a - x''}} \qquad (1)$$

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if the reaction is monomolecular;

$$\frac{T'}{T''} = \frac{\frac{a^n - (a-x')^n}{a^n \cdot (a-x')^n}}{\frac{a^n - (a-x'')^n}{a^n \cdot (a-x'')^n}} \qquad (2)$$

if the reaction is polymolecular. On the assumption that A' is the starting point of the reaction we get

$$K' = \frac{1}{T' + T^\circ} ln \frac{a}{a-x}$$

and

$$K'' = \frac{1}{T'' + T^\circ} ln \frac{a}{a-x''} \qquad (3)$$

or, more general

$$K' = \frac{1}{T' + T^\circ} \cdot \frac{a^n - (a-x')^n}{a^n \cdot (a-x')^n}$$

and

$$K'' = \frac{1}{T'' + T^\circ} \cdot \frac{a^n - (a-x'')^n}{a^n \cdot (a-x'')^n} \qquad (4)$$

Equations (1), (2), (3), and (4) give

$$\frac{K'}{K''} = \frac{(T'' + T^\circ)T'}{(T' + T)T''}$$

or, if we take the determinations at regular intervals $T'' = 2T'$ and $\frac{K'}{K''} = \frac{2T' + T^\circ}{2T' + 2T^\circ}$. $K'' > K'$, unless $T^\circ = 0$ or *if there is any latency time the reaction velocity will increase.*

In the work of both Bredig and his coworkers[1, 2] and Senter[5] the formula $K = \frac{1}{t} ln \frac{a}{a-x}$ is used, which gives the reason for the increase in the K values. Waentig and Steche[4] use the modification $K = \frac{1}{t-t_0} ln \frac{a-x_0}{a-x}$ in which x_0 is the amount decomposed at a certain time T. They do not use the starting point in their calculations. To find out whether the constant K values obtained by these authors

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were due to the formula they used, I tested this formula on some experiments of Bredig and von Berneck and of Senter.

T	$a-x$	$0.4343\,K$ $\left(\text{From } \log\frac{a}{a-x}\cdot\right)$	$0.4343\,K$ $\left(\text{From } \log\frac{a-x_0}{a-x}\cdot\right)$
Values of K obtained (Bredig and von Berneck*).			
0	19.0	0.0147	
9	13.7	0.0158	0.0168
14	11.4	0.0159	0.0164
20	9.0	0.0162	0.0166
Values of K obtained (Bredig and von Berneck†).			
0	21.5		
6	19.7	0.0063	
15	16.1	0.0084	0.0098
30	11.5	0.0091	0.0097
50	6.85	0.0099	0.0099
Values of K obtained (Senter‡).			
0	46.4		
4.75	41.6	0.0100	
15.5	32.3	0.0102	0.0112
25	25.3	0.0112	0.0106
40.15	17.0	0.0114	0.0109
80	5.3	0.0127	0.0104

* Bredig, G., and von Berneck, R. M., *Z. physikal. Chem.*, 1899, xxxi, 291.
† Bredig, G., and von Berneck, R. M., *Z. physikal. Chem.*, 1899, xxxi, 297.
‡ Senter, G., *Z. physikal. Chem.*, 1903, xxliv, 280.

It is obvious that the formula $K = \frac{1}{t-t_0} ln \frac{a-x_0}{a-x}$ gives a constant K. There certainly are cases in which the use of this formula does not prevent the increase of reaction velocities; *e.g.*, the inversion of cane-sugar.[6] But I am inclined to believe that in most of the cases with catalase this increase was due to the properties of the formula with which the results were calculated.

[6] Henri, V., *Z. physikal. Chem.*, 1902, xxxix, 194.

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ASSOCIATIVE BACTERIAL ACTION IN THE PROPIONIC ACID FERMENTATION.*

By JAMES M. SHERMAN and ROSCOE H. SHAW.

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(Received for publication, January 13, 1921.)

Among the most interesting problems in bacteriology are those that have to do with the mutual relations of the organisms under natural conditions. Numerous notations of effects, both beneficial and detrimental, of one organism upon another are to be found in bacteriological literature. The beneficial action on a specific organism of the associative growth of another organism is well illustrated by such well known examples as the helpful effect on the tetanus bacillus of associated aerobes; and the increased vigor of the lactic acid fermentation when conducted in impure culture, which has been studied extensively by Marshall and his associates. Antagonisms, aside from those which may be readily explained upon a purely physical or chemical basis, have been noted in many connections. An example of this nature is the recent report of Smith and Smith[1] on the inhibiting action of the paratyphoid organism upon the gas production of *Bacterium coli*.

In the present paper we wish to call attention to a beneficial associative effect of greater magnitude than is frequently observed, and which involves the little studied propionic acid fermentation. The full details of this work will be included in a forthcoming paper on the propionic acid fermentation of lactose.

The propionic acid-producing organism used in these experiments was of the type which has been tentatively designated as *Bacterium acidi propionici* (*d*) and which has been shown to be the essential organism for the production of "eyes" and the characteristic flavor in Swiss cheese.[2] The other organisms employed were *Streptococcus*

* Published with the permission of the Secretary of Agriculture.

[1] Smith, T., and Smith, D. E., *J. Gen. Physiol.*, 1920, iii, 21.

[2] Sherman, J. M., *J. Bact.*, 1921, vi, in press.

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lacticus, *Lactobacillus casei*, and Cultures 45.3 and 45.4, two non-lactose-fermenting organisms which have not been identified nor subjected to detailed study.

The results of a typical experiment are given in Table I. The cultures were grown for 1 month at 30°C. in a medium containing 5 per cent lactose, 1 per cent peptone, and an excess of calcium carbonate. Similar results have been obtained in many different tests and with other organisms.

From these figures it is seen that this propionic organism is greatly aided in its action by the associated growth of certain other bacteria. In the cases of *Streptococcus lacticus* and *Lactobacillus casei* the

TABLE I.

Effect of Associative Action.

Inoculation.	Propionic acid.
	per cent
Propionic organism alone	0.081
" " + *Streptococcus lacticus*	0.470
" " + *Lactobacillus casei*	0.589
" " + Culture 45.3	0.559
" " + " 45.4	0.362

increased production of propionic acid might be assumed to be due to a greater utilization of the lactic acid produced by these bacteria than of the lactose, since the propionic organism is an active fermenter of lactates. Von Freudenreich and Jensen[3] showed that the volatile acids produced from milk by *Bacterium acidi propionici* (*a*), a feeble lactose fermenter, could be greatly increased by growing the organism in combination with lactic acid-forming organisms. However, our results obtained in other tests in which the fermentable material consisted of calcium lactate instead of lactose also showed a stimulating effect of *Lactobacillus casei* upon the propionic acid-producing organism. In this connection it is interesting to note that certain non-lactose-fermenting bacteria stimulate the propionic acid fermentation of lactose in the same way.

It is hoped that we may return to this problem at some future time in an effort to throw some light on the nature of the associative effect.

[3] von Freudenreich, E., and Jensen, O., *Landw. Jahrb.*, 1906, xx, 320.

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THE PHYSIOLOGICAL ZERO: AN EXPLANATION OF THE DEPARTURE FROM THE LINEAR GRAPH OF REACTION RATE VALUES AT THE LOWER TEMPERATURES.*

By JOSEPH KRAFKA, Jr.

With the Collaboration of R. P. Stevens and David F. Barrow.

(*From the Zoological Laboratory of the University of Georgia, Athens.*)

(Received for publication, January 25, 1921.)

Considerable controversy has arisen between investigators dealing with the influence of temperature upon physiological reaction rates. Some reactions are decidedly exponential, others clearly linear functions of the temperature. To the first class belong the results of Snyder (1913), on the rate of beat of the mammalian heart and those of Loeb and Northrop (1917) on the length of life of *Drosophila*. The work of Sanderson (1910), Peairs (1914) and others on insect development and that of Krogh (1914) on general metabolism establish the latter relationships.

Marked deviations from the linear curves have been noted at the upper and lower temperatures, making necessary the qualifying clause that the linear relations only held between the normal limits of growth. The departures at the upper temperatures have received an explanation. It is generally conceded that the linear is a modified exponential whose secondary factor increases more rapidly than the primary one at the higher temperatures.

The departures at the lower temperatures have only been noted in connection with methods for the calculation of the physiological zero. Thus if we were to apply Peairs' method for the determination of the physiological zero to the development of *Drosophila melanogaster* it would come at about 8°C. in the work of Loeb and Northrop (1917), and at 10°C. in my own experiments (Krafka (1919–20)).

* Contribution from the Zoological Laboratory of the University of Georgia, No. 1.

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Yet Plough reared larvae and pupae at 5°C. Similar deviations are apparent in all of Krogh's curves.

These deviations at the lower temperatures further support the hypothesis that the linear curve

$$y \propto x$$

is in reality a modified exponential curve

$$y \propto 2^x$$

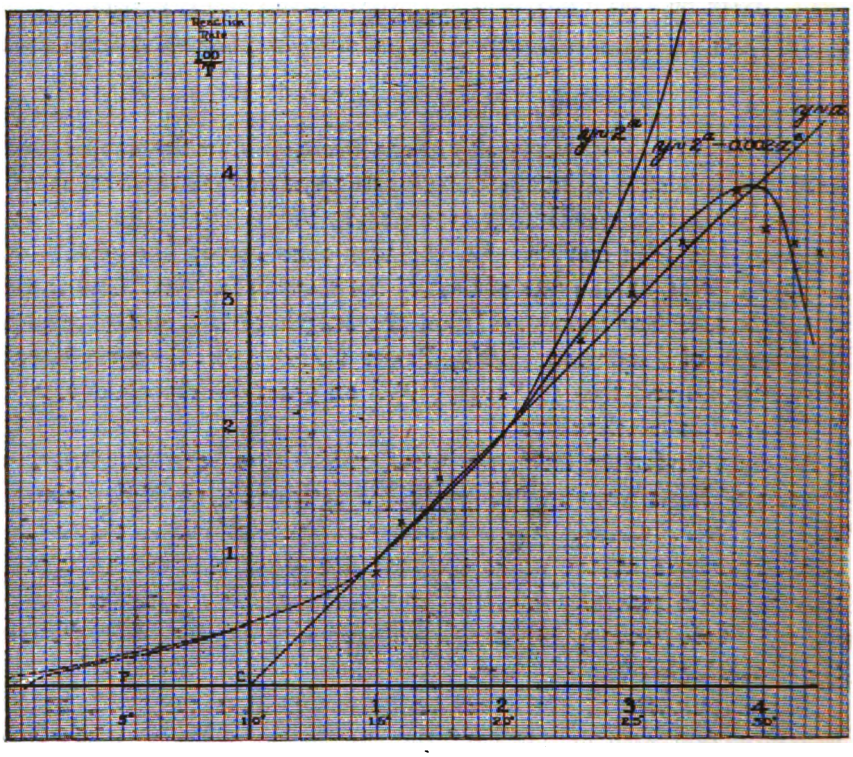

FIG. 1. Curves representing the relations between the temperature and the rate of development of the fruit fly, *Drosophila melanogaster*. The experimental points x fall along the linear curve $y \propto x$ between certain limits. Projecting this curve to the base line the physiological zero of Peairs is obtained at 10°C. The point P represents an observation establishing development below this by Plough. The curve $y \propto 2^x$ is a theoretical van't Hoff curve involving two experimental values, while the modified exponential is shown as the middle one at each end.

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for under these conditions they are normal and expected, since they represent only the continuation of the primary exponential.

Dr. R. P. Stephens and Dr. David F. Barrow have supplied me with an empirical formula from data on the rate of development of the fruit fly, *Drosophila melanogaster*, which fulfills the conditions for the flattening of a primary exponential into a linear with a sharp bend at the upper end.

$$y \propto 2^x - 0.002x^6$$

This corrective factor at the same time breaks the primary exponential at the lower temperatures so that a theoretical zero becomes possible.

While any number of mathematical functions could be devised whose graphs would fit the experimental points fairly well, the latter formula stated in general terms should have some practical value.

$$y \propto A^x - Bx^n$$

where A represents van't Hoff's constant, B and n calculated values to represent the divergence between the linear and exponential at the uppermost points.

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COMPARATIVE STUDIES ON RESPIRATION.

XVII. Decreased Respiration and Recovery.

By O. L. INMAN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, March 17, 1921.)

In a previous paper the writer[1] has shown that exposure to hypotonic or hypertonic solutions may greatly lower the production of carbon dioxide by *Laminaria agardhii*. The object of the present investigation was to determine whether the respiration would become normal when the plant was replaced in sea water. The work was done at Woods Hole, where material could be obtained under the most favorable conditions.

The normal rate of respiration was determined (in the manner previously described) and the tissue was exposed to the hypertonic or hypotonic solution for a definite time; it was then removed and its respiration was measured.[2] The piece of tissue was then replaced in running sea water. At varying intervals it was removed and the rate of respiration was determined. As in the previous investigation[1] these determinations were made in van't Hoff's solution in order to avoid the buffer effect of the sea water.

Fig. 1 shows that in strongly hypertonic sea water respiration steadily decreases and that the degree of recovery depends on the length of the exposure. After an exposure of five minutes recovery is practically complete: as the period of exposure is lengthened recovery is less and less complete and when the exposure amounts to 20 minutes there is no recovery whatever.

It is evident that the upper curves dip slightly after the tissues are returned to normal sea water. This is not seen in the experiments with hypotonic solutions and isotonic sodium chloride and may

[1] Inman, O. L., *J. Gen. Physiol.*, 1921, iii, 533.

[2] The measurement of respiration was always made with the tissues in the dark.

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perhaps be explained as a purely mechanical disturbance due to the great differences in osmotic pressure as the result of which the tissue must remain about 30 minutes in normal sea water before the readjustment is complete.

Fig. 2 shows that the effects of strongly hypotonic sea water are similar to those of strongly hypertonic, except that the lowering of respiration is not so pronounced. Here also we observe all degrees of recovery depending on the length of exposure to the solution.

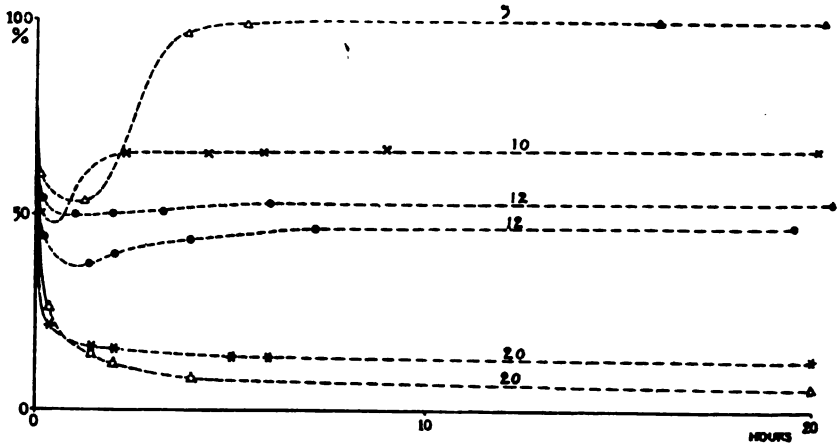

FIG. 1. Curves showing rate of respiration of *Laminaria* (expressed as per cent of the normal). The normal rate represents a change from pH 7.78 to 7.36 in from 1½ minutes to 2 minutes, depending upon the amount of material used. The solid lines show rate of respiration while tissue was exposed to hypertonic sea water (sp. gr. 1.130, $\Delta = -9.37$ approximately). The dotted lines show stages of recovery after the tissue was put back in normal sea water. Each curve represents a typical experiment. The figure attached to each recovery curve denotes the time (in minutes) of exposure to the solution of hypertonic sea water; thus the uppermost curve represents recovery, after an exposure of 5 minutes.

The question naturally arises as to what happens when these pieces of tissue are kept longer than 20 hours in running sea water. This was carefully investigated and it was found that every piece of tissue which had been exposed to hypertonic or hypotonic sea water died and disintegrated much sooner in running sea water than the normal piece of tissue kept in the same vessel and treated in the same manner except that it was not exposed to hypertonic or hypotonic solutions.

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Pieces of tissue that showed complete recovery were found to live longest, as expected, but none of these remained normal more than seven days while the untreated showed a normal rate of respiration after eighteen days. Those pieces that showed partial recovery were marked by a fall in the rate, usually at the end of three days, but in a few cases not until the end of five days. Where there was no recovery the pieces turned green and soon disintegrated.

A series of experiments was made in which pieces of *Laminaria* were placed in isotonic sodium chloride (0.52 M for Woods Hole sea water) and the respiration measured at the end of an hour. The

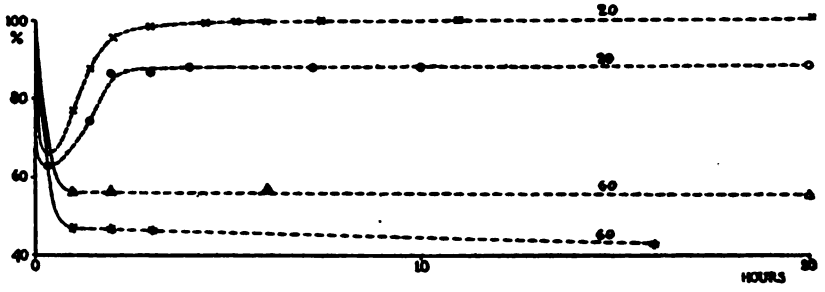

FIG. 2. Curves showing rate of respiration of *Laminaria* (expressed as per cent of the normal). The normal rate represents a change from pH 7.78 to 7.36 in from 1½ to 2 minutes, depending upon the amount of material used. The solid lines show rate of respiration while tissue was exposed to hypotonic sea water (sp. gr. 1.0023, $\Delta = -0.187$). The dotted lines show stages of recovery after the tissue was put back in normal sea water. Each curve represents a typical experiment. The figure attached to each recovery curve denotes the time (in minutes) of exposure to the solution of hypotonic sea water.

pieces were then returned to running sea water and at intervals shown by the points on dotted lines of Fig. 3, they were removed and the rate of respiration determined.

In this case some pieces of tissue were more affected than others by the same length of exposure (due to the thickness of the fronds, the temperature of the experiment, etc.), and it is noticeable that the greater the lowering of the respiration the less complete the recovery. Here also it was found that the treated pieces of tissue lived but a short time in running sea water as compared with the normal pieces.

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It is therefore evident that in all these experiments recovery may be incomplete and even where the tissue is kept for days under the most favorable circumstances there is no tendency in these cases for the recovery to complete itself. This offers a striking parallel to the experiments recently described by Osterhout[3] in which all degrees of recovery (depending upon the length of exposure) were observed when *Laminaria* was placed for a time in 0.52 M sodium chloride (and in other solutions)[4] and then replaced in sea water. In this case electrical conductivity was used as a criterion of recovery.

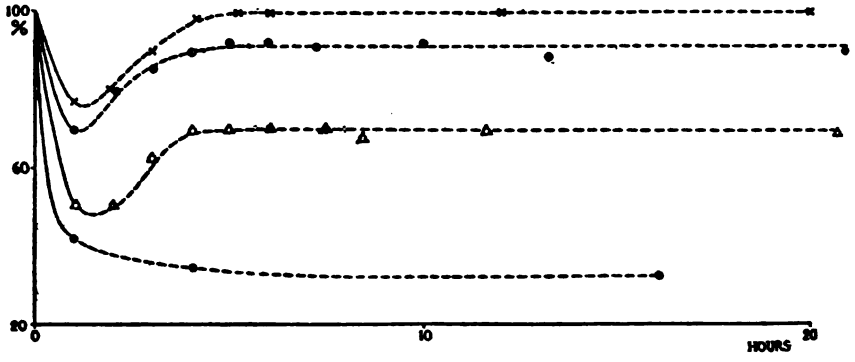

FIG. 3. Curves showing rate of respiration of *Laminaria* (expressed as per cent of the normal). The normal rate represents a change from pH 7.78 to 7.36 in from 1½ to 2 minutes, depending upon the amount of material used. The solid lines show rate of respiration during one hour of exposure to isotonic sodium chloride (0.52M for Woods Hole sea water). The dotted lines show stages of recovery after the tissue was put back in normal sea water. Each curve represents a typical experiment.

It is possible that when recovery is incomplete none of the cells are killed but that their respiration is permanently decreased. It is also possible that some cells continue to respire normally while others are killed and that it is the death of these cells which prevents complete recovery. This question must remain for future investigation.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920, iii, 15.

[4] Unpublished experiments by Osterhout on the electrical conductivity of *Laminaria* show incomplete recovery after exposure to hypo- and hypertonic solutions.

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DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

I. Membrane Potentials.

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I. INTRODUCTION.

The different physical properties of proteins, such as osmotic pressure, swelling, and viscosity, vary in a similar way under the influence of changes in the concentration of hydrogen ions or under the influence of neutral salts or under the influence of the valency of the ion in combination with the protein. This fact suggests that the variations of these three physical properties may have a common cause, so that if this cause were known for one of these properties it would, perhaps, be known for all of them. The clues tentatively suggested have thus far either proved wrong or inaccessible to quantitative tests.

In the last paper[1] the writer showed that a measurable potential difference is found when we separate a protein solution from a watery solution (free from protein) by a collodion membrane. When the protein in solution is a protein-acid salt (*e.g.*, gelatin chloride) the protein solution is positive and the water is negative; when the protein exists in the form of a metal proteinate the protein solution is negative and the watery solution positive. The turning point seems to lie at the isoelectric point. Quantitative measurements of these P.D. between gelatin chloride solutions and the outside watery solution with which the gelatin chloride solution was in osmotic equilibrium revealed the fact that these potential differences varied in a similar way as the osmotic pressure, the swelling, or the viscosity

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557.

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when a neutral salt was added or when the hydrogen ion concentration was altered or when the valency of the ion in combination with the protein changed. This in itself would have meant only the addition of another property varying in the same characteristic way as osmotic pressure, swelling, or viscosity if it had not been for the fact that it was possible to correlate the origin of this new property with Donnan's membrane equilibrium. Donnan's membrane equilibrium[2] is established when a membrane separates two solutions of electrolytes one of which has one ion for which the membrane is impermeable, while all the other ions can diffuse through the membrane. It is not necessary that this non-diffusible ion should be a colloid, it may just as well be a crystalloid; all that is required is that it be impossible for this ion to diffuse through the membrane. The protein ions generally satisfy this condition and a collodion membrane properly prepared is a membrane impermeable to a protein ion.

When equilibrium is established in such a system the distribution of the ions is not the same on both sides of the membrane and from thermodynamic considerations Donnan was able to develop the equations for the relative concentration of the different ions on opposite sides of the membrane at equilibrium. When a collodion bag filled with a 1 per cent gelatin chloride solution is dipped into a beaker containing a solution of HCl (without gelatin) of the same pH as that of the gelatin solution, the concentration of the hydrochloric acid becomes greater outside than inside the gelatin solution. The Donnan equilibrium demands that free acid be expelled from the gelatin solution into the outside solution and this actually occurs.[3]

Procter[4] has proposed an osmotic theory of the swelling of gelatin chloride on the assumption that the swelling is a purely osmotic phenomenon. He deduces from Donnan's theory that at the point

[2] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[3] This is possible on account of the fact that a gelatin chloride solution always contains free HCl and that there seems to be a chemical equilibrium inside the gelatin chloride solution between the free HCl, the gelatin chloride, and the non-ionogenic gelatin.

[4] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

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of equilibrium the relative distribution of HCl inside and outside the solid block of gelatin chloride is determined by the following equation:

$$x^2 = y\,(y + z)$$

where x is the concentration of H ions (and of Cl ions) outside the gelatin, y the concentration of the H (and Cl) ions of the free HCl inside the gelatin, and z the concentration of the Cl ions in combination with the gelatin ions. Since all quantities are positive, it follows that x must be greater than y; *i.e.*, the concentration of free HCl in the outside solution must be greater than in the gel. The writer has shown that exactly the same happens when we separate a solution of gelatin chloride from pure water by a collodion membrane.

This difference in the concentration of acid on the two sides of the membrane leads to a difference in P.D. at the boundary of the membrane.[5]

This can be proved by the fact that it is possible to calculate the P.D. of the system

$$- \left| \begin{matrix} \text{outside solution} \\ \text{(free from gelatin)} \\ \text{concentrated HCl} \end{matrix} \right| \begin{matrix} \text{collodion} \\ \text{membrane} \end{matrix} \left| \begin{matrix} \text{inside solution} \\ \text{(gelatin chloride)} \\ \text{dilute HCl} \end{matrix} \right| +$$

with a fair degree of accuracy on the basis of Nernst's well known logarithmic formula from the difference of the hydrogen ion concentration on the opposite sides of the membrane. On the basis of Nernst's formula for concentration cells the P.D. is at a temperature of 24°C. 0.059 $\log \frac{C_1}{C_2}$, where C_1 is the concentration of hydrogen ions inside the gelatin solution and C_2 the hydrogen ion concentration outside the gelatin solution. Hence we may substitute the value pH inside minus pH outside for the value $\log \frac{C_1}{C_2}$. (The term pH inside means the pH in the gelatin solution and the term pH outside means the pH in the outside solution with which the gelatin solution is in equilibrium.) This paper intends to show that if we multiply

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

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the value of pH inside minus pH outside by 59 we can calculate with a fair degree of accuracy the P.D. observed at 24°C. in millivolts when equilibrium between the gelatin chloride solution and the outside solution is established.

II. METHODS.

The potential differences were determined with the aid of a Compton electrometer giving a deviation of about 2 mm. on the scale for 1 millivolt at a distance of about 2 m. The gelatin solution was inside the collodion bag closed with a rubber stopper through which a funnel was introduced; the funnel was filled high enough with liquid to permit the electrode to dip into the gelatin solution. The collodion bag containing the solution of gelatin-acid salt (*e.g.*, gelatin chloride) dipped into the water with which the gelatin solution was in equilibrium. The water, therefore, contained always free acid and in certain experiments also salt solution when the nature of the experiment demanded this. The second electrode was introduced into this outside watery solution. Calomel electrodes with saturated KCl solution were used and the saturated KCl solutions were brought into contact with the outside and inside solutions through glass tubes. The ends of these tubes which dipped into the inside and outside solutions were drawn out into capillaries and bent upwards to prevent the influence of gravity on the diffusion of the saturated KCl solution into the inside and outside solutions. The only potential differences existing in this system were those on the opposite sides of the membrane.

III. The Influence of Neutral Salts on the Potential Difference between Gelatin Chloride Solutions and Outside Solutions.

1 gm. of isoelectric gelatin was made into a 1 per cent solution by either dissolving it in H_2O or in a solution of NaCl differing in molecular concentration from M/4,096 to 1 M. To every solution so much HCl was added that the pH of the solution was 3.5. Collodion bags of a volume of about 50 cc. were filled with this solution, each collodion bag being connected with a glass tube serving as manometer, as described in preceding publications. These collodion bags

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were dipped into beakers containing 350 cc. of HCl solution of pH 3.0. The HCl solutions in the beakers were made up in NaCl solutions of different concentrations and the concentration of the NaCl solution in the beaker was at the beginning of the experiment always identical with the concentration of the NaCl in the gelatin solution inside the collodion bag. The final measurements were made after 18 hours when the osmotic and the membrane equilibria were established. The osmotic pressure was a maximum (about 425 mm. water) in the gelatin chloride solution free from salt, and the osmotic pressure was the lower the higher the concentration of the salt added. This effect is represented in the upper curve of Fig. 1. The abscissae are the concentration of the NaCl solution and the ordinates the osmotic pressure. The curve shows that the osmotic pressure drops very rapidly with the increase in the concentration of NaCl.

The potential differences at the boundary of the inside and outside solutions were measured with a Compton electrometer as described and the values found are plotted on the second upper curve in Fig. 1. The scale for the ordinates was selected in such a way as to make the osmotic pressure ordinate and the ordinate for the P.D. coincide for a M/4,096 NaCl solution. The reader will notice that the two curves for osmotic pressure and P.D. coincide practically throughout which signifies that the P.D. and the osmotic pressure of the gelatin chloride solution undergo a similar depression under the influence of a neutral salt like NaCl.

We stated in the last paper that the observed P.D. is always equal to the P.D. calculated on the assumption that at equilibrium the P.D. is due to the difference in the pH inside and outside the collodion bag. The pH inside and outside were measured electrometrically and the results are contained in Table I.

On the assumption stated above we can calculate the P.D. in millivolts by multiplying the value pH inside minus pH outside by 59, and the values so obtained should agree with the observed P.D. Table II shows that this is true.

The two lower curves of Fig. 1 give the depressing effect of different concentrations of Na_2SO_4 on the osmotic pressure and on the P.D. of a 1 per cent gelatin chloride solution of pH 3.5. Everything was the same as in the preceding experiment, except that Na_2SO_4 was sub-

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stituted for NaCl. The two lower curves in Fig. 1 show that the depressing effect of Na_2SO_4 on the osmotic pressure and the P.D. of a gelatin chloride solution is very similar and, moreover, that the depressing effect of Na_2SO_4 on both properties is more than twice (in reality nearly eight times) as great as that of NaCl. This influence

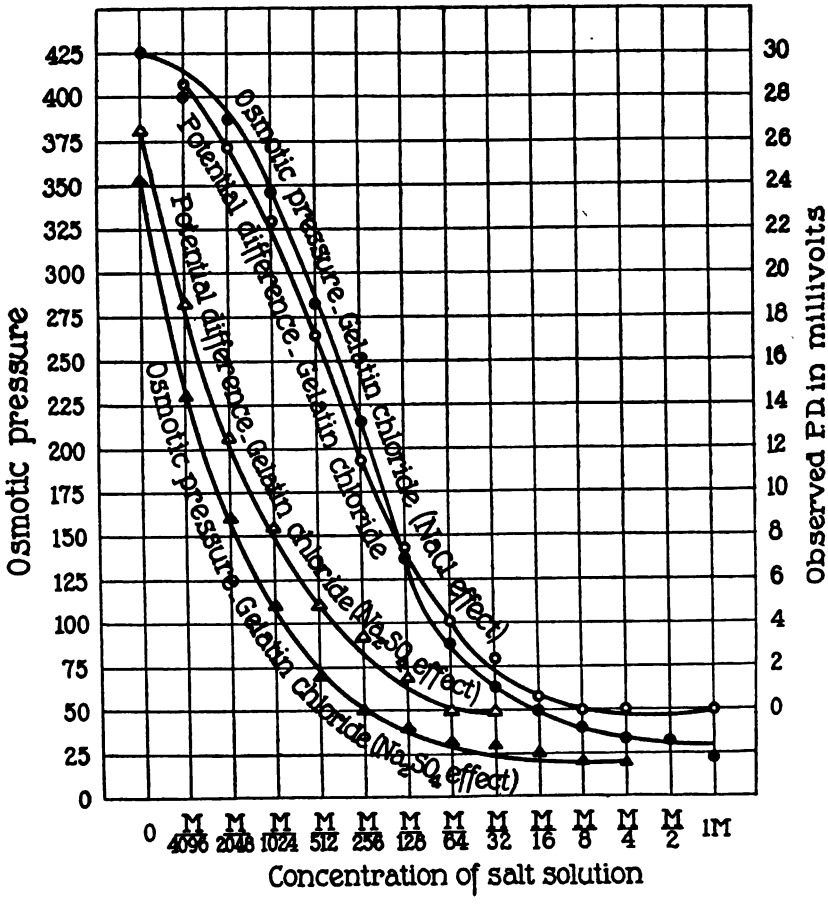

FIG. 1. Depressing effect of NaCl and Na_2SO_4 on osmotic pressure and P.D. of gelatin chloride solutions. Abscissae represent the concentration of salt solution, ordinates the osmotic pressure and P.D. in millivolts respectively. The depressing effect of neutral salts on osmotic pressure and P.D. is similar but not identical.

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TABLE I.

Influence of Concentration of NaCl on pH Inside Minus pH Outside at Equilibrium.

	Concentration of NaCl.													
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
pH inside.....	3.60	3.55	3.52	3.46	3.41	3.36	3.31	3.27	3.22	3.30	3.32	3.32	3.28	3.19
pH outside.....	3.09	3.08	3.09	3.11	3.13	3.16	3.20	3.20	3.18	3.28	2.30	3.30	3.26	3.19
pH inside minus pH outside...	0.51	0.47	0.43	0.35	0.28	0.20	0.11	0.07	0.04	0.02	0.02	0.02	0.02	0.00

TABLE II.[6]

Depressing Effect of NaCl on P.D. at Equilibrium.

Molecular concentration of NaCl.	P. D. observed.	P. D. calculated.
	millivolts	*millivolts*
0		+29.9
M/4,096	+28.6	+27.6
M/2,048	+25.7	+25.2
M/1,024	+22.3	+20.6
M/512	+17.1	+16.4
M/256	+11.4	+11.7
M/128	+ 7.4	+ 6.5
M/64	+ 4.0	+ 4.1
M/32	+ 2.3	+ 2.3
M/16	+ 0.6	+ 1.2
M/8	0.0	+ 1.2
M/4	0.0	+ 1.2
M/2	− 1.7	+ 1.2
1 M	0.0	0.0

[6] The sign of the observed P.D. was apparently, but not in reality, the reverse of the sign of the calculated P.D. This was due to the difference in the arrangement of the hydrogen electrodes in both cases. In the "observed" P.D. the membrane (serving as a hydrogen electrode) was between the concentrated and dilute HCl, while in the "calculated" values the P.D. was obtained from the potentiometric determinations of the pH. In this latter case the two hydrogen electrodes were separated by a concentrated and a dilute solution. The "observed" P.D. was hence between two solutions of different concentrations while in the "calculated" values we measured the P.D. between two electrodes. In our tables the apparent (but not real) reversal of sign due to the difference in arrangement of the hydrogen electrodes in the two cases is corrected.

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of the valency of anion in the case of gelatin-acid salt has been discussed in preceding papers. The question now arises: Does the value of the difference in the pH inside minus pH outside vary similarly as the values of the observed P.D. and the observed osmotic pressure, and do we get values approximating the observed P.D. if we multiply the value pH inside minus pH outside by 59? Table III gives the values for pH inside and outside and also the difference, pH inside minus pH outside, at the point of equilibrium (*i.e.*, after 18 hours).

The values for the P.D. calculated by multiplying the values for pH inside minus pH outside by 59 agree remarkably well with the observed values for P.D.

TABLE III.

Influence of Concentration of Na_2SO_4 on pH Inside Minus pH Outside at Equilibrium.

	Concentration of Na_2SO_4.											
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
pH inside	3.54	3.41	3.35	3.32	3.29	3.30	3.33	3.38	3.41	3.41	3.37	3.29
pH outside	3.07	3.12	3.14	3.17	3.20	3.24	3.30	3.35	3.38	3.38	3.36	3.28
pH inside minus pH outside	0.47	0.29	0.21	0.15	0.09	0.06	0.03	0.03	0.03	0.03	0.01	0.01

We will consider as a third case the influence of a salt of the type $CaCl_2$ on the osmotic pressure and the P.D. of a gelatin chloride solution. It had been shown in a preceding paper that the depressing effect of $CaCl_2$ is always about twice as great as that of an equimolecular concentration of NaCl and that hence the whole effect of $CaCl_2$ is due exclusively to the Cl ion. Fig. 2 shows that the depressing effects of $CaCl_2$ on the osmotic pressure and the P.D. run closely parallel and that the depressing effect of $CaCl_2$ on P.D. is about the same as that of a NaCl of twice the molecular concentration. Table V gives the influence of $CaCl_2$ on the values of pH inside minus pH outside, and shows that the agreement between the observed and the calculated P.D. is quite close.

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It follows from these experiments, first, that the curves representing the influence of neutral salts on P.D. are similar to the curves representing the influence of the same salts on the osmotic pressure of

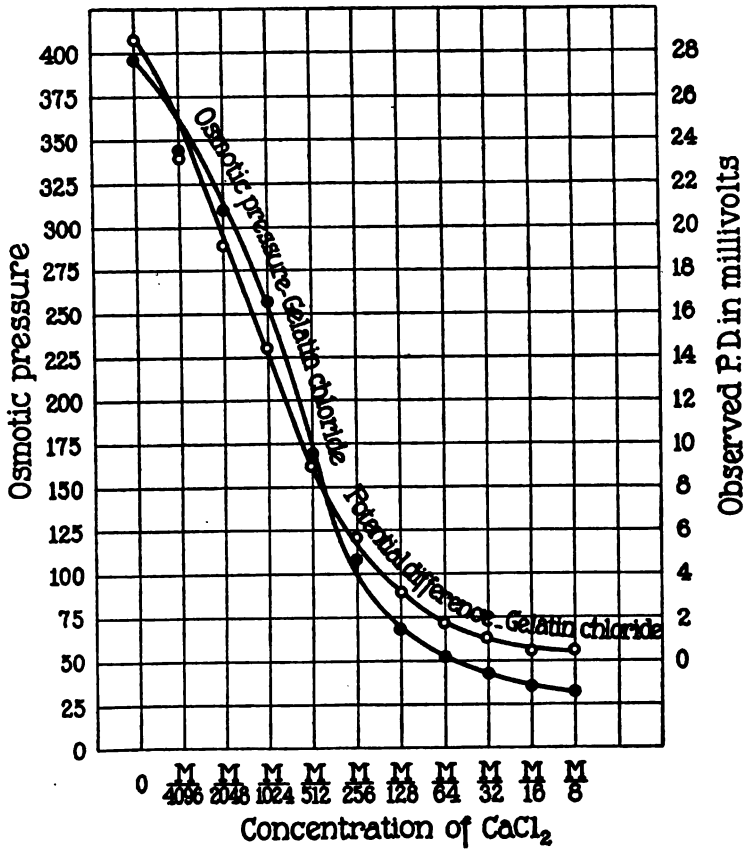

FIG. 2. Similarity of depressing effect of $CaCl_2$ on osmotic pressure and P.D. of gelatin chloride solution.

the same gelatin chloride solutions; and second, that the P.D. calculated by multiplying the values of pH inside minus pH outside by 59 agrees quite closely with the observed P.D.

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TABLE IV.

Depressing Effect of Na_2SO_4 on P.D. at Equilibrium.

Molecular concentration of Na_2SO_4.	P. D. observed.	P. D. calculated.
	millivolts	*millivolts*
0	+26.5	+27.6
M/4,096	+18.6	+17.0
M/2,048	+12.5	+12.3
M/1,024	+ 8.4	+ 8.8
M/512	+ 4.7	+ 5.3
M/256	+ 3.4	+ 3.5
M/128	+ 1.5	+ 1.7
M/64	0.0	+ 1.7
M/32	0.0	+ 1.7
M/16	0.0	+ 1.7
M/8	0.0	+ 0.6
M/4	0.0	+ 0.6

TABLE V.

Influence of Concentration of $CaCl_2$ on pH Inside Minus pH Outside and on P.D. at Equilibrium.

	Concentration of $CaCl_2$.										
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside.....	3.55	3.45	3.41	3.36	3.30	3.28	3.26	3.25	3.25	3.25	3.22
pH outside....	3.05	3.06	3.09	3.12	3.15	3.17	3.20	3.22	3.24	3.24	3.22
pH inside minus pH outside.....	0.50	0.39	0.32	0.24	0.15	0.11	0.06	0.03	0.01	0.01	0.0
P.D. calculated (millivolts)..	+29.5	+23.0	+18.9	+14.1	+8.8	+6.5	+3.5	+1.8	+0.6	+0.6	0.0
P.D. observed (millivolts)..	+28.6	+23.4	+19.2	+14.5	+9.1	+5.7	+3.1	+1.8	+1.1	+0.5	+0.5

IV. Influence of Neutral Salts on the Potential Differences between Solid Gelatin and Outside Solution.

It is well known that the addition of neutral salt depresses the swelling of solid gelatin chloride or of any gel with a pH different from that of the isoelectric point. This is intelligible on the basis of

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Procter's osmotic theory of swelling which assumes that the swelling of a gel is due to the osmotic pressure inside the gel, the surface of the gel acting as a membrane impermeable to gelatin ions but permeable to the H or Cl or salt ions. On this assumption we should expect the establishment of a Donnan equilibrium between the liquid inside the gel and the outside liquid, and Procter has proved that this is the case. We should also expect a P.D. at the surface of the gel between the solution inside and outside the gel, due to the Donnan equilibrium, and we should, moreover, expect that the P.D. could be calculated from the value of pH inside minus pH outside as described for the experiments on osmotic pressure. This expectation is confirmed except that the results are liable to be less regular than in the case of the osmotic pressure experiments. This is probably due to accidental errors, one being possibly that some of the gel dissolves in the outside solution so that the outside solution is no longer free from gelatin. Such an error does not exist when we separate a gelatin solution from the outside solution by a collodion membrane.

Our method was as follows. 1 gm. of powdered gelatin of pH 7.0 was brought to the isoelectric point by treatment with M/128 acetic acid and subsequent washing with cold H_2O as described in previous papers. The doses of powdered wet isoelectric gelatin were then put into 200 cc. of M/128 HCl made up in different concentrations of NaCl varying from 0 to M/8, and the degree of swelling was measured in terms of the height of a column of the powdered gelatin in 100 cc. graduates after equilibrium was established (after two hours). From this the volume of the isoelectric gelatin was deducted. Fig. 3 shows that the swelling diminished with the quantity of salt added. The mass was put on a filter and allowed to drain thus separating the gelatin from the supernatant liquid. The gelatin was then melted and its pH determined electrometrically. This gave us the pH inside, and by determining the pH of the outside solution the values of the pH inside minus pH outside were ascertained. The liquid gelatin was then poured into cylinders with a small bent side tube attached, which was also filled with gelatin. The gelatin was then cooled and allowed to set to a jelly. The P.D. between the solid jelly and the outside solution was then determined by pushing one electrode into the gel while the other electrode was introduced into the

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beaker containing the outside solution. The latter solution was brought into contact with the gelatin in the side tube, the latter

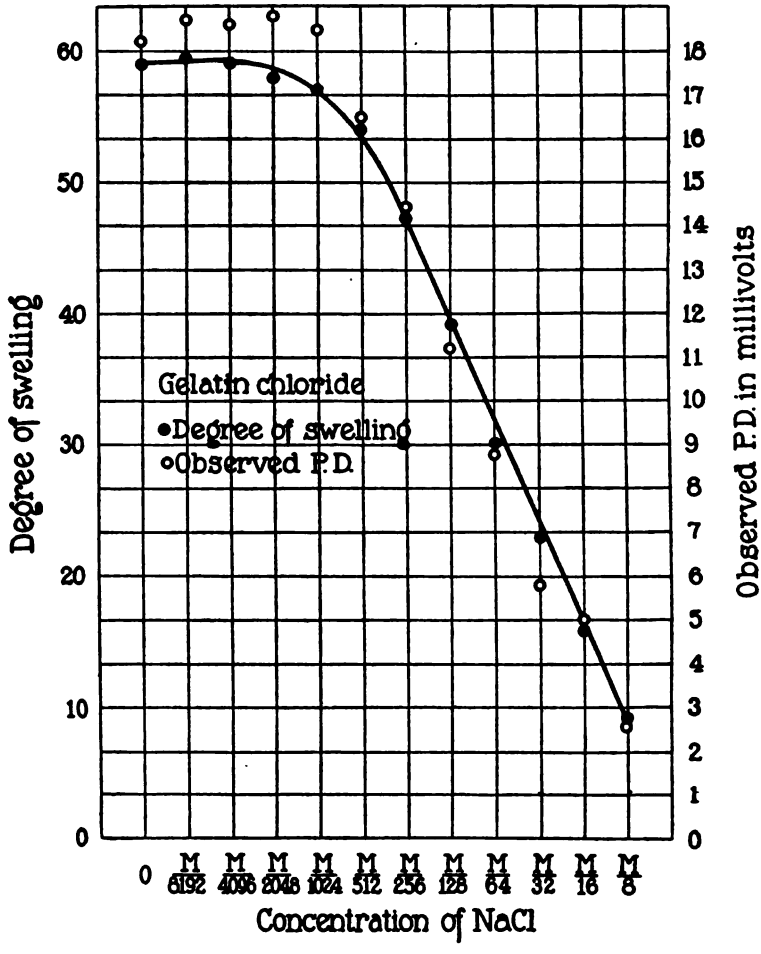

FIG. 3. Similarity of depressing effect of NaCl on swelling and P.D. of solid gelatin chloride.

dipping into the beaker. Table VI gives a comparison of the observed P.D. and those calculated by multiplying the values pH inside minus pH outside by 59. The agreement is satisfactory.

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TABLE VI.

Influence of Concentration of NaCl on pH Inside Minus pH Outside and on P.D. of Solid Gelatin Chloride at Equilibrium.

	Concentration of NaCl.											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside	2.71	2.70	2.69	2.69	2.70	2.69	2.65	2.61	2.57	2.56	2.54	2.50
pH outside	2.38	2.38	2 37	2.37	2.38	2.38	2.39	2.40	2.42	2.43	2.45	2.46
pH inside minus pH outside	0.33	0.32	0.32	0.32	0.32	0.31	0.26	0.21	0.15	0.13	0.09	0.04
P.D. calculated (millivolts)	+19.4	+18.8	+18.8	+18.8	+18.8	+18.2	+15.3	+12.4	+8.8	+7.6	+5.3	+2.4
P.D. observed (millivolts)	+18.2	+18.7	+18.6	+18.8	+18.5	+16.4	+14.3	+11.2	+8.8	+5.8	+5.0	+2.6

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The same experiment was repeated with Na_2SO_4 as a salt instead of with NaCl. A comparison of Fig. 4 with Fig. 3 shows that Na_2SO_4 depresses the swelling of gelatin chloride more powerfully than NaCl

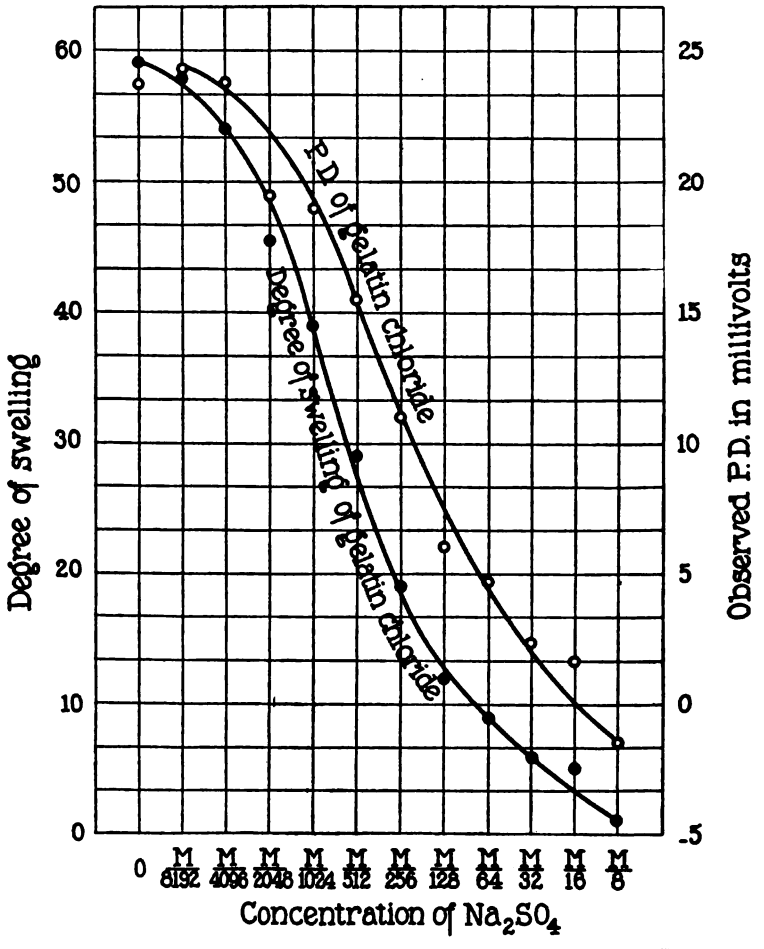

FIG. 4. Similarity of depressing effect of Na_2SO_4 on swelling and P.D. of solid gelatin chloride.

and that it depresses also the P.D. more powerfully than is done by NaCl. Table VII gives the pH inside minus pH outside, and a comparison of the P.D. calculated and observed. The agreement is again satisfactory.

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TABLE VII.

Influence of Concentration of Na_2SO_4 on pH Inside Minus pH Outside and on P.D. of Solid Gelatin Chloride at Equilibrium.

	Concentration of Na_2SO_4.											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside	2.69	2.69	2.70	2.70	2.70	2.67	2.67	2.67	2.68	2.75	2.82	2.88
pH outside	2.40	2.38	2.37	2.38	2.42	2.43	2.48	2.53	2.61	2.71	2.82	2.92
pH inside minus pH outside	0.29	0.31	0.33	0.32	0.28	0.24	0.19	0.14	0.07	0.04	0.00	−0.04
P.D. calculated (millivolts)	+17.0	+18.3	+19.5	+19.0	+16.5	+14.1	+11.2	+8.2	+4.1	+2.5	0.0	−2.5
P.D. observed (millivolts)	+23.7	+24.3	+23.7	+19.5	+19.0	+15.5	+11.0	+6.0	+4.8	+2.4	+1.8	−1.5

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We therefore come to the conclusion that the depressing influence of the neutral salts on the P.D. between solid gelatin and a surrounding aqueous solution runs parallel to the depressing effect of the same salts on the swelling of gelatin and that the P.D. can be calculated with the aid of Nernst's formula, on the assumption that the difference in the hydrogen ion concentration inside the gel and in the outside solution determines the P.D.

V. The Influence of the Hydrogen Ion Concentration of Gelatin Solutions on the P.D.

The osmotic pressure of 1 per cent solutions of originally isoelectric gelatin varies with the pH of the gelatin solution and with the valency of the ion in combination with the gelatin. This is illustrated in Fig. 5 where the ordinates represent the observed osmotic pressures of 1 per cent solutions of gelatin chloride, gelatin phosphate, gelatin oxalate, and gelatin sulfate. The osmotic pressure rises steeply as soon as the pH becomes less than 4.7, reaching a maximum at pH of about 3.6, and then drops steeply with a further decline of pH. Moreover, it is obvious that the curves for gelatin chloride and phosphate, both possessing a monovalent anion, are identical, that the curve for gelatin oxalate, which has mainly a monovalent anion at the pH under discussion, is almost but not quite as high as that for gelatin chloride, but that the curve for gelatin sulfate (possessing a bivalent anion) is only about half as high as that for gelatin chloride. The P.D. of 1 per cent solutions of these four salts contained in collodion bags were measured against outside aqueous solutions (without gelatin) after equilibrium was reached (*i.e.* after about 18 hours). The bags contained about 50 cc. of the gelatin solution while the beaker contained 350 cc. H_2O with so much acid that the pH of the water was at the beginning of the experiment always identical with the pH of the gelatin solution; and for the outside solution the same acid was used as for the gelatin solution. Fig. 6 gives the curves for the value of the P.D. observed. The following points of similarity between the two sets of curves for osmotic pressure (Fig. 5) and P.D. (Fig. 6) are noticeable. Both sets of curves rise from the isoelectric point with a lowering of the pH until they reach a maximum; this maxi-

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mum is, however, not identical. For P.D. it varies between 3.6 and 4.0, while for osmotic pressure it lies near 3.6.[7] With a further fall in pH both sets of curves show approximately the same steep drop.

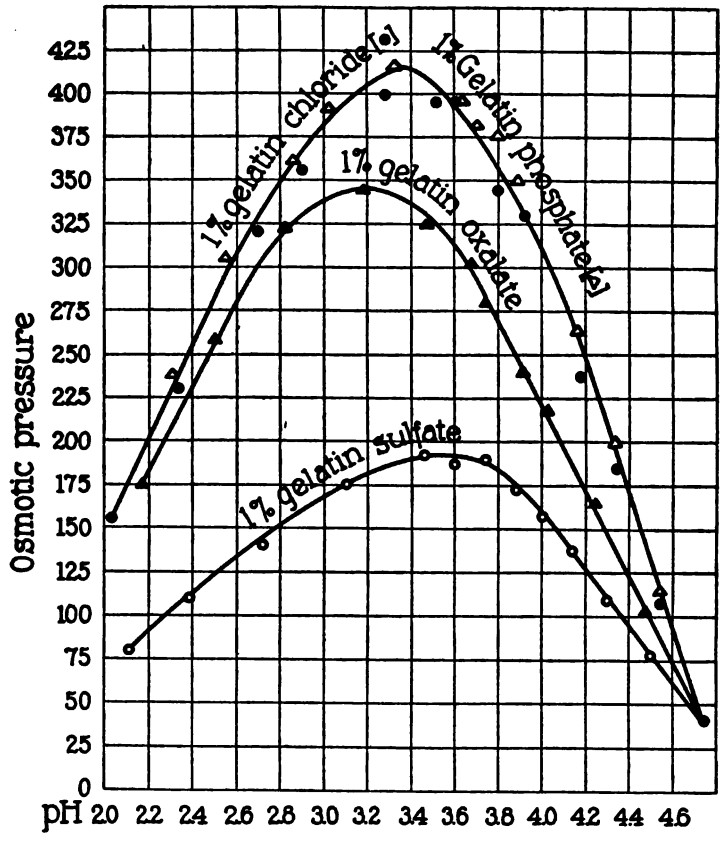

FIG. 5. Influence of pH and valency of anion on osmotic pressure of solutions of different gelatin-acid salts.

The second point of similarity is the influence of valency. The curves for P.D. (Fig. 6) are practically the same for gelatin chloride and gelatin phosphate, while the curve for P.D. is considerably lower in the case of gelatin sulfate.

[7] It may be stated incidentally that the maximum for the viscosity of gelatin solution also lies at a different pH, namely 3.0, from the maximum for osmotic pressure, which lies at pH 3.6.

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These experiments offer an excellent opportunity to test our theory that the P.D. can be calculated with a fair degree of accuracy from the values of pH inside minus pH outside on the basis of Nernst's formula. Tables VIII, IX, and X show that this is true. The upper

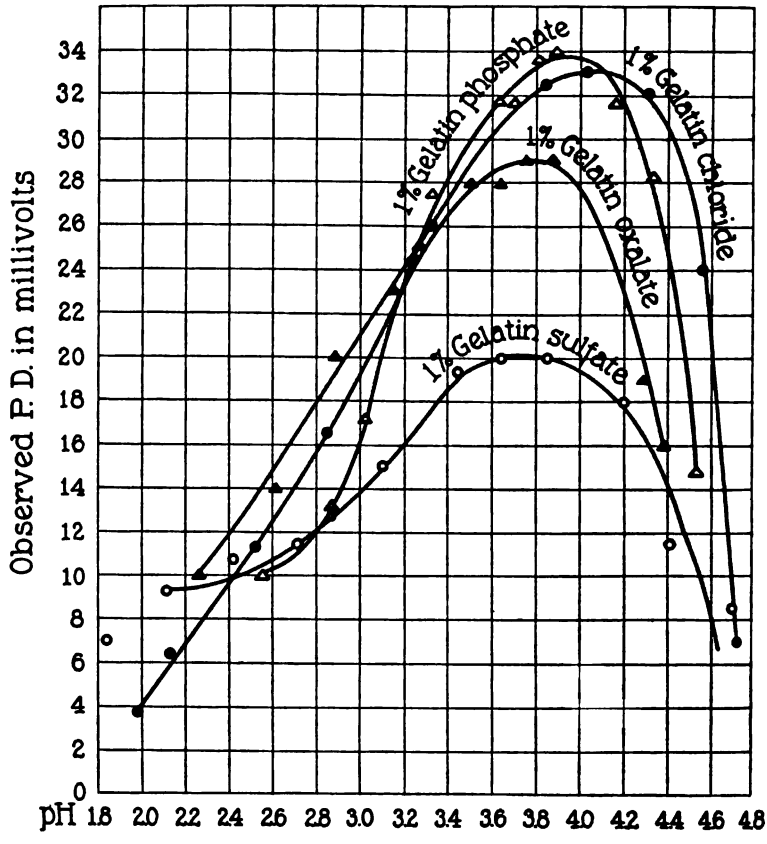

FIG. 6. Influence of pH and valency of anion on P.D. of solutions of different gelatin-acid salts. The curves in Fig. 6 are similar to (but not identical with) those in Fig. 5.

two horizontal rows give the pH inside and outside, the third horizontal row gives the difference pH inside minus pH outside, and the fourth row gives the P.D. calculated in millivolts by multiplying the values pH inside minus pH outside by 59. The last horizontal row

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TABLE VIII.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on the P.D. of Gelatin Chloride Solutions at Equilibrium.

	Cc. 0.1 N HCl added to 100 cc. 1 per cent isoelectric gelatin.											
	1	2	4	6	8	10	12.5	15	20	30	40	50
pH inside	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
pH inside minus pH outside	0.42	0.53	0.59	0.59	0.46	0.44	0.32	0.24	0.13	0.10	0.07	0.04
P.D. calculated (millivolts)	+24.7	+31.0	+34.5	+34.5	+27.0	+25.8	+18.8	+14.0	+7.6	+5.9	+4.1	+2.3
P.D. observed (millivolts)	+24.0	+32.0	+33.0	+32.5	+26.0	+24.5	+16.5	+11.2	+6.4	+4.8	+3.7	+2.1

TABLE IX.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on P.D. of Gelatin Phosphate Solutions at Equilibrium.

	Cc. M/10 H_3PO_4 added to 100 cc. 1 per cent isoelectric gelatin.													
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
pH inside minus pH outside	0.09	0.44	0.54	0.58	0.54	0.52	0.48	0.44	0.36	0.28	0.20	0.14	0.09	0.07
P.D. calculated (millivolts)	+5.3	+25.8	+31.7	+34.0	+31.7	+30.5	+28.0	+25.8	+21.2	+16.4	+11.7	+8.2	+5.3	+4.1
P.D. observed (millivolts)	+5.7	+27.0	+29.0	+30.0	+30.6	+29.6	+26.5	+24.4	+22.3	+17.7	+15.6	+11.4	+9.9	+7.3

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TABLE X.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on P.D. of Gelatin Sulfate Solutions at Equilibrium.

	Cc. 0.1 N H_2SO_4 added to 100 cc. 1 per cent isoelectric gelatin.													
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
pH inside minus pH outside	0.15	0.32	0.35	0.38	0.35	0.31	0.27	0.24	0.17	0.12	0.07	0.06	0.04	0.03
P.D. calculated (millivolts)	+8.8	+18.8	+20.5	+22.2	+20.5	+18.1	+15.8	+14.0	+10.0	+7.0	+4.1	+3.5	+2.4	+1.8
P.D. observed (millivolts)	+6.3	+16.3	+18.4	+19.0	+19.0	+17.4	+15.8	+13.7	+10.5	+8.4	+7.4	+5.8	+4.7	+3.7

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gives the observed P.D. in millivolts. The agreement between observed and calculated P.D. is sufficiently close.

VI. Hydrogen Ion and Chlorine Ion Potentials.

The equation for the equilibrium condition between gelatin chloride solution and water is as stated above,

$$x^2 = y\,(y + z)$$

where x is the concentration of H and Cl ions in the outside solution, y the concentration of the H and Cl ions of the free HCl inside the gelatin chloride solution, and z the concentration of the Cl ions held by the gelatin.

If we write this equation in the form

$$\frac{y}{x} = \frac{x}{y + z}$$

$\frac{y}{x}$ is the ratio of hydrogen ion concentration inside over the hydrogen ion concentration outside; and $\frac{x}{y + z}$ is the ratio of the concentration of the chlorine solution outside over the chlorine solution inside. Since

$$\log \frac{y}{x} = \text{pH inside minus pH outside}$$

and

$$\log \frac{x}{y + z} = \text{pCl outside minus pCl inside}$$

it follows that

$$\text{pH inside minus pH outside} = \text{pCl outside minus pCl inside}$$

It was easy to put this consequence of Donnan's theory to a test and some of the experiments described in the preceding chapter were selected for this purpose. Inside the collodion bag was a 1 per cent solution of gelatin chloride of different pH, outside water. After 18 hours equilibrium was established between inside and outside solutions and the pH as well as the pCl were ascertained. The pCl

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was determined in two different ways in the two experiments; in one experiment it was determined with the potentiometer, in the other it was determined in the gelatin chloride solution by titration with NaOH according to the method described in a preceding paper.[8] Both methods of determining the pCl led to the result that the values pCl outside minus pCl inside were for the same solution at the point of equilibrium equal to the value pH inside minus pH outside (within the limits of accuracy of the experiments). The pCl outside was identical with the pH outside, since the outside solution contained only free HCl. The values of pH were all determined potentiometrically.

TABLE XI.

Experiment 1. pCl determined by titration.

pH of gelatin chloride solution at equilibrium	4.13	3.69	3.30	3.10	2.92	2.78	2.46	2.26	2.01	1.76
pH inside minus pH outside	0.56	0.58	0.50	0.49	0.44	0.44	0.33	0.23	0.15	0.10
pCl outside minus pCl inside	0.48	0.51	0.59	0.44	0.44	0.38	0.35	0.22	0.15	0.11

Experiment 2. pCl determined electrometrically.

pH of gelatin chloride solution at equilibrium	4.04	3.92	3.78	3.61	3.46	3.16	2.73	2.36	2.04	1.73
pH inside minus pH outside	0.60	0.62	0.66	0.55	0.50	0.43	0.30	0.20	0.12	0.07
pCl outside minus pCl inside	0.55	0.60	0.57	0.50	0.53	0.38	0.32	0.17	0.12	0.07

Nernst's formula leads therefore to the same theoretical P.D. regardless of the fact whether we calculate the P.D. on the basis of the difference pH inside minus pH outside or pCl outside minus pCl inside. It is also obvious that both assumptions lead to the same sign of charge of the gelatin chloride solution. If we assume that the P.D. is determined by differences in the hydrogen ion concentration the outside solution is concentrated and the inside solution dilute; if the P.D. is determined by differences in the concentration of the Cl ions the inside solution is concentrated and the outside solution dilute. Since the common ion is positive in the former and negative in the latter case, the gelatin solution becomes positive in both cases.

[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

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outside solution.		inside solution.
$-H^+$ concentrated $-Cl^-$ dilute	membrane	H^+ dilute $+$ Cl^- concentrated $+$

The facts of this last chapter prove beyond doubt that the equation $x^2 = y(y + z)$ is the correct expression for the Donnan membrane equilibrium between acid-salts of proteins with monovalent anion and water. The close analogy between the variation of the membrane potentials and the other osmotic properties of solutions of protein-acid salts, such as osmotic pressure, viscosity, and swelling, suggests that an attempt be made to derive the variations of these latter properties directly from the equation for the Donnan equilibrium. This has already been done by Procter for the swelling of gelatin chloride.

SUMMARY.

1. It is shown that a neutral salt depresses the potential difference which exists at the point of equilibrium between a gelatin chloride solution contained in a collodion bag and an outside aqueous solution (without gelatin). The depressing effect of a neutral salt on the P.D. is similar to the depression of the osmotic pressure of the gelatin chloride solution by the same salt.

2. It is shown that this depression of the P.D. by the salt can be calculated with a fair degree of accuracy on the basis of Nernst's logarithmic formula on the assumption that the P.D. which exists at the point of equilibrium is due to the difference of the hydrogen ion concentration on the opposite sides of the membrane.

3. Since this difference of hydrogen ion concentration on both sides of the membrane is due to Donnan's membrane equilibrium this latter equilibrium must be the cause of the P.D.

4. A definite P.D. exists also between a solid block of gelatin chloride and the surrounding aqueous solution at the point of equilibrium and this P.D. is depressed in a similar way as the swelling of the gelatin chloride by the addition of neutral salts. It is shown that the P.D. can be calculated from the difference in the hydrogen ion concentration inside and outside the block of gelatin at equilibrium.

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5. The influence of the hydrogen ion concentration on the P.D. of a gelatin chloride solution is similar to that of the hydrogen ion concentration on the osmotic pressure, swelling, and viscosity of gelatin solutions, and the same is true for the influence of the valency of the anion with which the gelatin is in combination. It is shown that in all these cases the P.D. which exists at equilibrium can be calculated with a fair degree of accuracy from the difference of the pH inside and outside the gelatin solution on the basis of Nernst's logarithmic formula by assuming that the difference in the concentration of hydrogen ions on both sides of the membrane determines the P.D.

6. The P.D. which exists at the boundary of a gelatin chloride solution and water at the point of equilibrium can also be calculated with a fair degree of accuracy by Nernst's logarithmic formula from the value pCl outside minus pCl inside. This proves that the equation $x^2 = y\,(y + z)$ is the correct expression for the Donnan membrane equilibrium when solutions of protein-acid salts with monovalent anion are separated by a collodion membrane from water. In this equation x is the concentration of the H ion (and the monovalent anion) in the water, y the concentration of the H ion and the monovalent anion of the free acid in the gelatin solution, and z the concentration of the anion in combination with the protein.

7. The similarity between the variation of P.D. and the variation of the osmotic pressure, swelling, and viscosity of gelatin, and the fact that the Donnan equilibrium determines the variation in P.D. raise the question whether or not the variations of the osmotic pressure, swelling, and viscosity are also determined by the Donnan equilibrium.

The measurements referred to in this paper were made by the writer's technical assistants, Mr. M. Kunitz and Mr. N. Wuest, to whom the writer wishes to express his indebtedness.

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DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

II. Osmotic Pressure.

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I. INTRODUCTION.

It was pointed out in two preceding papers[1] that the curves representing the influence of electrolytes on the membrane potentials between gelatin solutions and water at the point of equilibrium show a marked similarity to the curves representing the influence of the same electrolytes on osmotic pressure, swelling, and viscosity of gelatin. There was no doubt left that the Donnan equilibrium accounted quantitatively for these variations in the case of membrane potentials[2] and the question therefore arose whether the same equilibrium condition can account also for the corresponding variations of the osmotic pressure of protein solutions. An attempt was, therefore, made to calculate the osmotic pressures on the basis of the Donnan equilibrium in order to find out whether the curves for the calculated values would show the characteristics of the curves representing the observed values for osmotic pressures. The depressing effect of the addition of a neutral salt on the osmotic pressure of colloidal solutions was predicted by Donnan and offered no theoretical difficulty.[3] The real test was whether the Donnan equilibrium would be able to account for the peculiar curves obtained when the observed osmotic pressures of 1 per cent solutions of gelatin-acid salts are plotted as a function of the pH.

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[3] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

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Fig. 1 shows a set of such curves where the abscissæ are the pH of the gelatin solution at equilibrium, and the ordinates the observed osmotic pressures. There are two outstanding peculiarities in these curves, namely, first, that they all rise from a minimum at pH 4.7 (the isoelectric point of gelatin) until they reach a maximum at pH

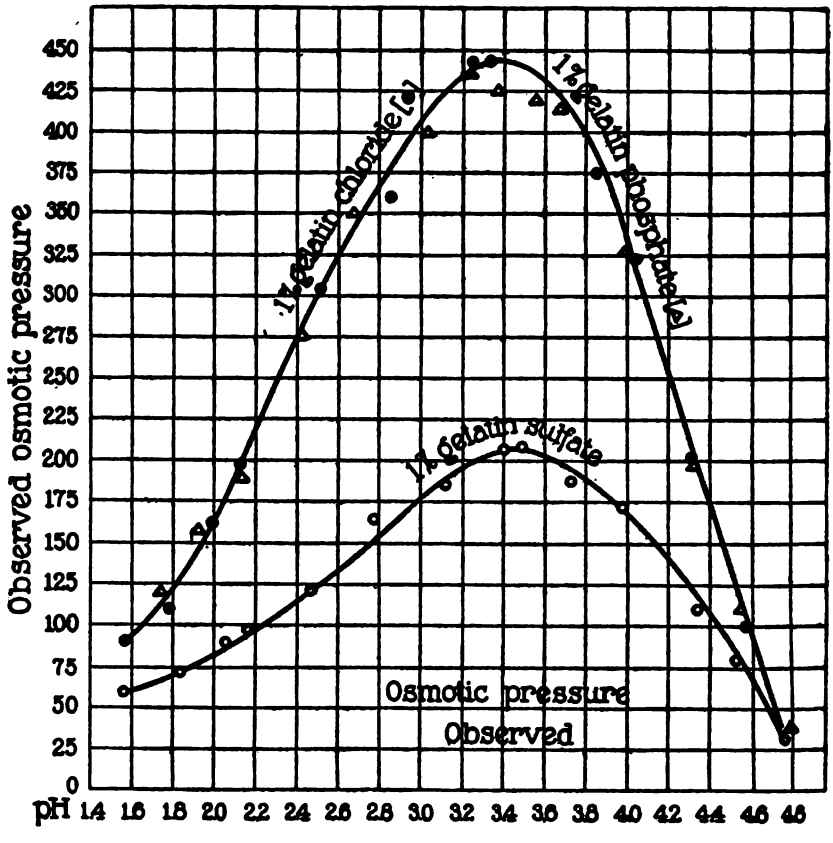

FIG. 1. Curves representing the influence of pH and valency of anion on osmotic pressure of solutions of gelatin-acid salts containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The curves for gelatin chloride and gelatin phosphate are identical since the anions, Cl and H_2PO_4, of these two gelatin salts are monovalent. The curve for gelatin sulfate is less than half as high as the curve for the two other salts because the anion of gelatin sulfate is bivalent. Both curves rise from the isoelectric point at 4.7 to a maximum at pH about 3.4 or 3.5, and then drop rapidly again.

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about 3.5, and then drop again; and second, that only the valency of the ion in combination with a protein influences its osmotic pressure (or degree of swelling, etc.), while the specific nature of the ion aside from its valency has no influence. The latter fact is really the crucial point which decides between colloid chemistry and classical physical chemistry. If only the valency of the ion in combination with a protein is of importance and if gelatin-acid salts of the same pH and the same concentration of originally isoelectric gelatin have the same osmotic pressure, provided the anion of the gelatin-acid salt has the same valency, a suspicion must arise that we are dealing with some equilibrium condition for which classical physical chemistry is able to account. The writer has shown in preceding papers that gelatin chloride, nitrate, acetate, succinate, tartrate, citrate, and phosphate have at the same pH and the same concentration of originally isoelectric gelatin the same osmotic pressure; and it was shown by titration curves that the anion in all these salts is monovalent. The titration curves show also that the anion in gelatin sulfate is bivalent and we have found that the osmotic pressure of gelatin sulfate is less than half that of gelatin chloride or phosphate at the same pH and for the same concentration of originally isoelectric gelatin.[4]

Fig. 1 illustrates this valency effect in the observed osmotic pressure. The curves for the observed osmotic pressure of gelatin chloride and gelatin phosphate are identical while the curve for gelatin sulfate is considerably lower.

It is the purpose of this paper to show that we can calculate with a fair degree of accuracy the osmotic pressure of gelatin solutions on the assumption of the validity of Donnan's equilibrium equation and the validity of van't Hoff's theory of osmotic pressure.

II. Theoretical Data.

A gelatin chloride solution contains free hydrochloric acid, gelatin chloride (which dissociates electrolytically like any other salt in watery solution), and non-ionogenic protein molecules. A 1 per cent gelatin chloride solution of about pH 3.5 is in equilibrium with a

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391; *Science*, 1920, lii, 449.

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HCl solution (free from protein) of a pH of about 3.0, both solutions being separated by a collodion membrane.

Let y be the concentration of the H and Cl ions of the free HCl inside a gelatin chloride solution (containing 1 gm. of originally isoelectric gelatin in 100 cc.), z the concentration of the Cl ions held by the gelatin ions, and a the sum of the gelatin ions and non-ionized molecules of gelatin. For the sake of simplification we assume complete electrolytic dissociation of the gelatin chloride and of the HCl. In this case the real osmotic pressure of the inside solution is determined by

$$2y + z + a$$

Since, however, the outside solution is at equilibrium not H_2O but a HCl solution—in the example selected of about pH 3.0—the *observed* osmotic pressure is the difference between the osmotic pressure of the inside solution against H_2O and the osmotic pressure of the outside solution.

Let x be the concentration of the H ions in the outside solution, then the osmotic pressure of the outside solution is determined by $2x$.

Hence the observed osmotic pressure P_o of the gelatin chloride solution is determined by

$$P_o = 2y + z + a - 2x$$

P_o is observed experimentally, y can be calculated from the pH inside, and x from the pH outside.

z can be calculated from Donnan's equilibrium equation in the form given it by Procter[5]

$$x^2 = y\,(y + z) \qquad (1)$$

$$z = \frac{(x + y)\ (x - y)}{y}$$

where x, y, and z have the significance stated above. We have seen in the preceding paper that this equation leads to correct results in regard to the P.D.

[5] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

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a is unknown, and we therefore can only calculate for the present the values of

$$2y + z - 2x$$

If we express the theoretical osmotic pressure of a grammolecular solution in terms of mm. pressure of a column of H_2O we get (with correction for a temperature of 24°C.)

$$22.4 \times 760 \times 13.6 \times \frac{297}{273} = 2.5 \times 10^5$$

In other words, a theoretical pressure of 2.5 mm. H_2O corresponds to a concentration of 10^{-5} N. In the following tables all concentrations are expressed in terms of 10^{-5} N and hence we only need to multiply the values for $2y + z - 2x$ given in our tables by 2.5 to obtain the calculated osmotic pressure of the gelatin solution (neglecting the osmotic pressure of the gelatin ions and molecules).

Equation (1) holds in the case of solutions of all gelatin-acid salts with monovalent anion; *i.e.* gelatin chloride, acetate, phosphate, tartrate, citrate, etc. When, however, the anion of a gelatin-acid salt is divalent, as in the case of gelatin sulfate, the equilibrium equation becomes one of the third degree. If x be the hydrogen ion concentration of the outside solution, the concentration of the SO_4 ion in the outside solution becomes $\frac{x}{2}$. If y be the concentration of the H ions of the free sulfuric acid in the inside solution, $\frac{y}{2}$ is the concentration of the SO_4 ions of the free acid inside the gelatin sulfate solution. In the case of gelatin chloride z represented the concentration of chlorine ions in combination with the gelatin; hence $\frac{z}{2}$ will represent the concentration of SO_4 ions in combination with the same number of gelatin ions.

The equilibrium equation, therefore, assumes in the case of gelatin sulfate the following form

$$x^2 \cdot \frac{x}{2} = y^2 \frac{(y + z)}{2} \qquad (2)$$

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From equation (2) follows

$$z = \frac{x^2 - y^2}{y^2}$$

The osmotic pressure of the gelatin solution should therefore be calculated from the following values (omitting the share of the osmotic pressure due to the gelatin)

$$\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x$$

III. The Calculated Curves.

Solutions containing 1 gm. of originally isoelectric gelatin in 100 cc. and containing different quantities of acid were prepared. Collodion bags cast in the form of Erlenmeyer flasks of 50 cc. volume were filled with the 1 per cent solution of a gelatin-acid salt and put into a beaker containing 350 cc. of H_2O. In order to accelerate the establishment of the equilibrium between inside and outside solutions a certain amount of acid was added to the outside water (*e.g.*, HCl in the experiments with gelatin chloride, H_3PO_4 in the experiments with gelatin phosphate, etc.). Each Erlenmeyer flask was closed with a rubber stopper perforated by a glass tube serving as a manometer. All this was described in more detail in previous publications.

In Fig. 2 are plotted the values of the calculated osmotic pressures for 1 per cent solutions of gelatin chloride, gelatin phosphate, and gelatin sulfate, and Tables I, II, and III give the data on the basis of which the curves in Fig. 2 are calculated. The experiments from which these calculations were made are identical with the experiments from which the curves for the observed osmotic pressures in Fig. 1 were plotted. The abscissæ in Fig. 2 are the pH in the inside solution at the point of equilibrium, the ordinates are the values for osmotic pressure calculated on the basis of the Donnan equilibrium as discussed before. The reader will notice that the three curves plotted in Fig. 2 show not only the same qualitative characteristics as the curves for the observed osmotic pressures in Fig. 1, but show them almost quantitatively; except that a correction for the value of osmotic pressure due to the gelatin particles itself may have to be

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TABLE I.

Gelatin Chloride.

Observed and Calculated Osmotic Pressures of Gelatin Chloride Containing 1 gm. of Originally Isoelectric Gelatin in 100 cc. Solution at Equilibrium.

pH inside	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
$y = C_H$ inside $\times 10^5$	2.7	4.9	9.3	14.1	46.8	56.2	141	302	741	1023	1622	2692
$x = C_H$ outside $\times 10^5$	7.2	16.6	36.3	54.9	135.0	155.0	295	524	1000	1288	1905	2951
$z = \frac{(x+y)(x-y)}{y}$	16.5	51.4	132.5	200.0	343.0	372.0	477	608	609	600	612	544
$2y + z - 2x$	7.5	28.0	78.5	118.4	166.6	174.4	169	164	91	70	46	26
Observed osmotic pressure	100	202	322	375	443	442	360	303	198	162	110	90
Calculated osmotic pressure, neglecting osmotic pressure of protein	19	70	196	296	416	436	422	410	227	175	115	65

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TABLE II.

1 Per Cent Gelatin Phosphate.

Observed and Calculated Osmotic Pressures at Equilibrium.

pH inside	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
$y = C_H$ inside $\times 10^5$	1.6	2.9	4.9	10.5	20.9	27.5	41.7	57.5	95.5	213.8	380.2	758.6	1202	1820
$x = C_H$ outside $\times 10^5$	2.0	7.9	16.9	39.8	72.4	91.2	125.9	158.5	218.8	407.4	602.6	1047.0	1479	2138
$z = \frac{(x+y)(x-y)}{y}$	0.9	18.6	53.3	140.0	228.0	231.0	338.0	380.0	405.0	556.0	575.0	686.0	617	690
$2y + z - 2x$	0.1	8.6	31.3	81.4	125.0	103.6	169.6	178.0	158.0	169.0	130.0	109.0	63	54
Observed osmotic pressure	34	111	199	328	416	420	426	436	401	350	275	190	158	121
Calculated osmotic pressure, neglecting osmotic pressure of protein		22	77	203	310	258	423	445	395	420	324	273	157	135

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TABLE III.

1 Per Cent Gelatin Sulfate.

Observed and Calculated Osmotic Pressure at Equilibrium.

pH inside	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
$y = C_H$ inside $\times 10^5$	1.7	3.0	4.6	10.4	18.6	32.3	38.9	75.9	166	339	692	871	1445	2692
$x = C_H$ outside $\times 10^5$	3.1	6.3	10.2	25.1	41.7	66.0	72.4	131.8	245.5	447	813	1000	1585	2884
$z = \frac{x^3 - y^3}{y^2}$	8.3	24.7	45.8	136	191.5	243	212	322	390	435	433	449	466	620
$\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x$	2	7.35	14.5	46	64	71	55.8	77	77	55	37	31	23	20
Observed osmotic pressure	33	79	110	172	188	208	208	185	164	122	98	89	72	61
Calculated osmotic pressure, neglecting osmotic pressure of protein	5	18.5	36	115	160	178		192	192	138	92.5	77.5	57.5	50

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added, a point which will be discussed in the next chapter. What is of importance here is the following. The curves for osmotic pressure calculated on the basis of the Donnan equilibrium and plotted

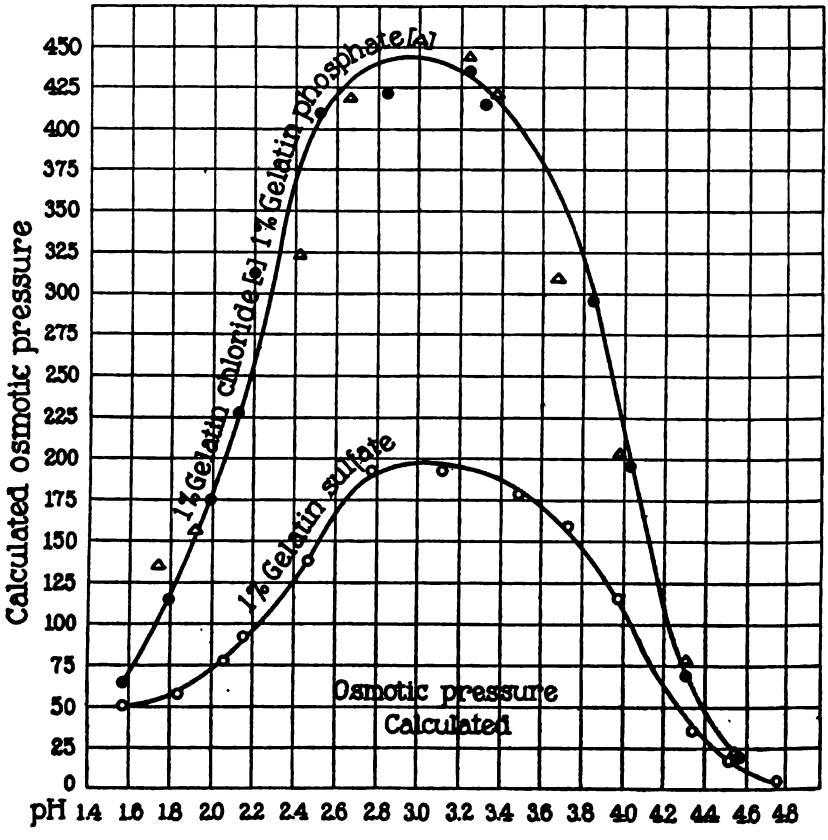

FIG. 2. Calculated curves of osmotic pressure taken from the data of the experiments represented in Fig. 1. The calculation is made on the basis of the validity of Donnan's theory of membrane equilibrium. The calculations lead to curves resembling the curves in Fig. 1 in all essential points, in regard to valency effect of the anion, as well as in regard to influence of pH. (See legend in Fig. 1.)

in Fig. 2 resemble the curves for the osmotic pressure observed in the same experiments represented in Fig. 1 in the following essential points.

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1. The curve for the calculated osmotic pressure of gelatin chloride is identical with the curve for the calculated osmotic pressure of gelatin phosphate, and the same is true for the two corresponding curves representing the observed osmotic pressures (Figs. 1 and 2).

2. The curve for the calculated osmotic pressure of gelatin sulfate is a little less than half as high as the curves for the calculated osmotic pressures of gelatin chloride and gelatin phosphate; and the same is true for the curves representing the observed osmotic pressures of gelatin sulfate and gelatin chloride.

3. All the curves in Figs. 1 and 2 rise from a minimum at pH 4.7, reach a maximum (which lies at pH 3.4 or 3.5 for the observed, and at 3.0 for the calculated curves), and then drop again as steeply as they rose on the other side. Moreover, the absolute values of observed and calculated osmotic pressures agree almost quantitatively, a fact which will be discussed more fully in Chapter V.

It may be added that the curve for the calculated values of the osmotic pressure of gelatin oxalate solutions agrees also with the curve for the observed values of the osmotic pressure of solutions of the same gelatin salt, both being slightly lower than the curves for gelatin chloride.

We can therefore say that (with the exception of two minor discrepancies to be discussed further on) the Donnan equilibrium accounts not only qualitatively but almost quantitatively for (*a*) *the valency effect of the anion with which the gelatin is in combination;* (*b*) *for the effect of the pH.*

Thus two of the most puzzling problems of the colloid chemistry of proteins seem to find their solution on the basis of classical physical chemistry.

IV. The Presumable Osmotic Pressure of Gelatin Particles.

The question now arises what the possible share of the protein particles in the osmotic pressure may be. Different concentrations of gelatin phosphate from 2 per cent to ½ per cent were prepared, all having a pH of 3.5. The gelatin phosphate solutions were put into Erlenmeyer flasks of 50 cc. volume, connected with a glass tube serving as a manometer as described, and these flasks were put into beakers containing 350 cc. of H_2O, the pH of which was brought at

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the beginning of the experiment to 3.5 through the addition of H_3PO_4. When the bags containing gelatin phosphate solutions are put into water the latter diffuses rapidly into the gelatin solution thereby lowering the concentration of the gelatin solution. To avoid this error so much gelatin phosphate solution was poured into each bag and glass tube that at the beginning of the experiment the liquid reached already to about that level which from preceding experiments we knew the gelatin solution would reach in the manometer at the point of osmotic equilibrium. All experiments were made in duplicate. In addition to the osmotic pressure we measured the pH inside and outside after equilibrium was reached. From these latter data the osmotic pressure due to the H and PO_4 ions could be calculated, being equal to

$$(2y + z - 2x) \times 2.5 \text{ mm } H_2O$$

By deducting this value from the observed osmotic pressure in each case it was hoped to obtain a rational value for the share of the protein particles in the observed osmotic pressure. Table IV gives the results.

The reader's attention is called to the last two rows of figures (Table IV) giving the difference between the observed and the calculated osmotic pressures, since if this difference actually represents the osmotic pressure due to the gelatin particles, the figures should be in direct proportion to the concentration of the gelatin. The experiments were all made in duplicate to give some idea of the magnitude of error and it is obvious that the error may be considerable, 25 per cent or more, because the errors in the observed and the calculated values are additive. Thus the "difference" is for $\frac{3}{4}$ per cent solution in one case 92, in the other 61, a variation of 50 per cent! If we take this into consideration we may conclude that the differences between the observed and the calculated osmotic pressures are compatible with the idea that the difference is the value for the osmotic pressure due to the gelatin particles in solution.

This would lead us to the conclusion that the osmotic pressure due to the gelatin particles in a 1 per cent solution (of originally isoelectric gelatin) of gelatin phosphate of pH 3.60 is about 100 mm. H_2O. Since the osmotic pressure of 1 grammolecule is about 250,000 mm.

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TABLE IV.

Influence of Concentration of Gelatin Phosphate of pH of About 3.6 on the Osmotic Pressure. (All Experiments Were Made in Two Sets.)

	Concentration of gelatin in per cent.									
	2	2	1½	1½	1	1	½	½	¼	¼
pH inside at equilibrium	3.64	3.66	3.60	3.60	3.65	3.66	3.60	3.60	3.61	3.62
pH outside at equilibrium	3.02	3.02	3.02	3.01	3.12	3.11	3.14	3.12	3.21	3.19
$y = C_H$ inside $\times 10^5$	22.9	21.9	25.1	25.1	22.4	21.9	25.1	25.1	24.6	24.0
$x = C_H$ outside $\times 10^5$	95.5	95.5	95.5	97.7	75.9	77.6	72.4	75.9	61.7	64.6
$z = \frac{(x+y)(x-y)}{y}$	375	395	338	355	235	253	184	204	130	150
$2y + z - 2x$	230	248	197	210	128	142	89	102	56	69
Observed osmotic pressure	860	860	715	680	420	445	314	316	186	186
Calculated osmotic pressure (ignoring gelatin)	576	620	493	523	320	355	222	255	140	172
Difference (osmotic pressure due to gelatin)	284	240	222	157	100	90	92	61	46	14
Mean	262		190		95		73		26	

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H_2O and since 1 liter of a 1 per cent solution of gelatin contains 10 gms. of gelatin, the molecular weight of gelatin should be expected to be in the neighborhood of 25,000. The experiment just described for gelatin phosphate was repeated for gelatin chloride, with similar results.

According to Dakin's[6] recent analyses gelatin contains 1.4 per cent phenylalanine. Since 1 molecule of gelatin cannot contain less than 1 molecule of phenylalanine and since the molecular weight of this

TABLE V.

Influence of Concentration of Albumin Chloride of pH of About 3.4 on the Osmotic Pressure.

	Concentration of Egg Albumin in per cent.					
	4	3	2	1	½	¼
pH inside at equilibrium.....	3.34	3.32	3.38	3.40	3.40	3.40
pH outside at equilibrium....	2.98	2.97	3.07	3.14	3.19	3.24
$y = C_H$ inside $\times 10^6$.....	45.7	47.9	41.7	39.8	39.8	39.8
$x = C_H$ outside $\times 10^6$.....	104.7	107.2	85.1	72.4	64.5	57.5
$z = \frac{(x+y)(x-y)}{y}$.....	194.0	192.0	132.0	92.0	64.6	43.3
$2y + z - 2x$.....	76	74	45	27	15	8
Observed osmotic pressure....	776	$555 + x$	375	163	75	36
Calculated osmotic pressure (ignoring albumin).....	190	185	113	67	39	20
Difference (osmotic pressure due to albumin).....	586	$370 + x$	262	96	36	16

amino-acid is 165 the lowest possible molecular weight of gelatin is 11,800. If a molecule of gelatin contains two molecules of phenylalanine, the molecular weight should be about 23,600. This would be approximately the figure we might expect from the data of Table IV on the assumption that the differences in the last two rows may be considered to be the values of the osmotic pressure of the protein particles.

[6] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

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A similar experiment was made with different concentrations of solutions of the chloride of crystalline egg albumin. The original pH of the albumin chloride solution was 3.5 and that of the outside solution 3.0. After equilibrium was established the pH both inside and outside was slightly changed as is shown in Table V. The osmotic pressures for $\frac{1}{4}$ to 4 per cent solutions of albumin chloride were measured and calculated for $2y + z - 2x$. The difference, which should be the osmotic pressure of the albumin particles in solution, is found in the last row. It is almost identical with the difference found for gelatin chloride for the same concentration of gelatin.

V. Difference between the Curves for Calculated and Observed Values.

The curves representing the values for calculated osmotic pressures differ in one or two respects from the curves representing the values for the observed osmotic pressures. These differences are not great but they are constant and can therefore not be due to an accidental error. Fig. 3 shows the difference between the curves for the observed and the calculated osmotic pressures in the case of a gelatin chloride solution containing 1 gm. of originally isoelectric gelatin in 100 cc. If we start with the ascending branch of the two curves of Fig. 3, we notice that the observed osmotic pressures for pH 4.4 to pH 3.8 are about 100 mm. higher in each case than the calculated pressures. It may be a mere accident but 100 mm. happens to be the approximate value for the osmotic pressure of 1 gm. of gelatin in 100 cc. and if the gelatin particles participate in the osmotic pressure of solutions of gelatin salts our calculated values should be about 100 mm. lower than the observed values for the same pH in the case of a 1 per cent gelatin solution. Near the summit the difference becomes a little less but we have seen that we may expect such irregularities on account of experimental error. Besides at pH 3.4 the concentration of the gelatin solution was diminished by about 20 per cent on account of diffusion of water into the gelatin bag. The difference in the ascending branch of the observed and calculated values occurs in every experiment.

The second constant difference between the curves for observed and calculated osmotic pressures lies in the fact that the drop in the calculated curves begins at a lower pH than the drop for the curves of

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observed values. This or both discrepancies may be due to a constant experimental error or they may find their explanation in the influence of one or more factors not taken into consideration in our calculations.

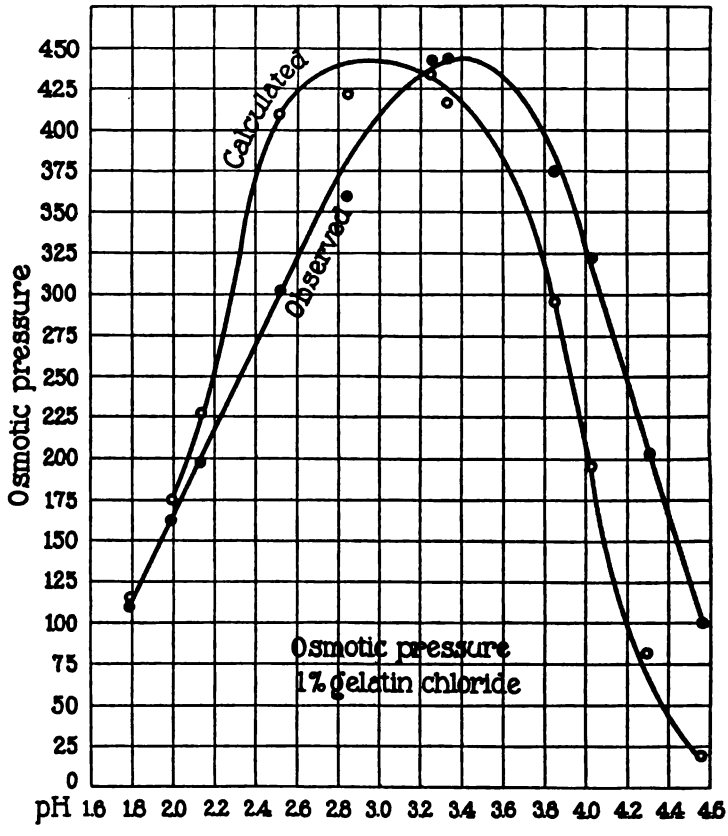

FIG. 3. Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin chloride solutions.

The calculated and observed curves for gelatin phosphate are identical with those for gelatin chloride given in Fig. 3, and we therefore omit these curves.

Fig. 4 gives the curves for observed and calculated values for gelatin sulfate. It seemed of interest to calculate the osmotic pres-

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sures from some of our older experiments on the osmotic pressure of crystalline egg albumin (containing 1 gm. of isoelectric albumin in 100 cc. solution). Fig. 5 gives a comparison of the curves for the

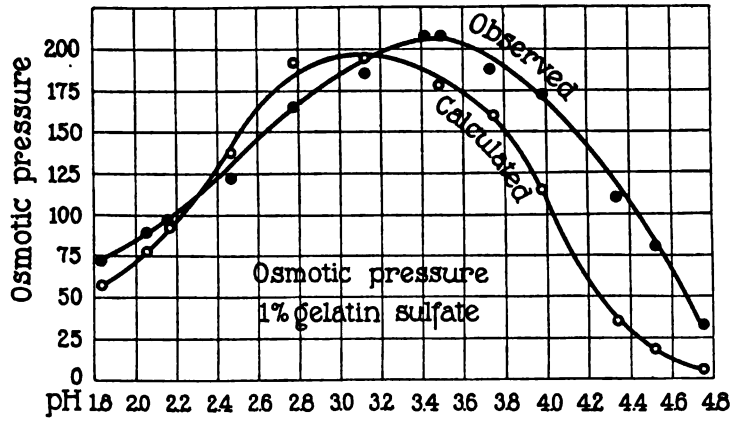

FIG. 4. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent gelatin sulfate.

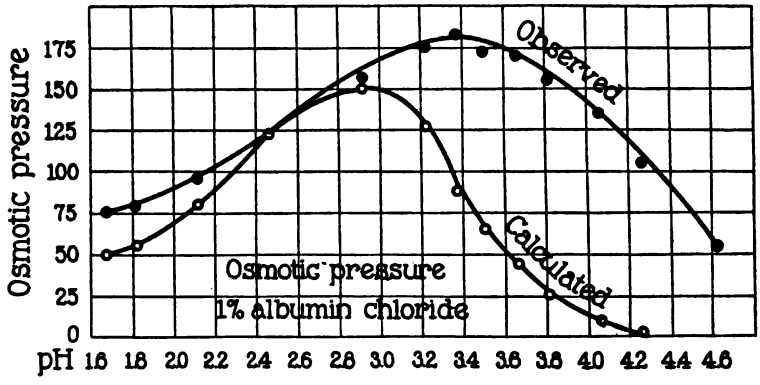

FIG. 5. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent crystalline egg albumin chloride.

observed and calculated osmotic pressures of 1 per cent albumin chloride and Fig. 6 the curves for observed and calculated osmotic pressures of 1 per cent albumin sulfate. In Fig. 6 appears the constant difference between the curves for observed and calculated

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values which the neglect of the protein value in the calculated curves postulates.

Procter assumes in his theory of swelling that the protein particles do not participate in the osmotic pressure inside the gel, and the writer is willing to admit that the same assumption may be necessary for the osmotic pressure of the protein solutions. The fact that the maximal observed osmotic pressure of gelatin solutions agrees with the maximal pressure calculated, without regard to the possible osmotic pressure caused by the protein ions, seems to agree with Procter's view. In this case, we should have to say that the curves for the observed values coincide with the curves for the calculated values if the latter curves are moved to the right, parallel with themselves.

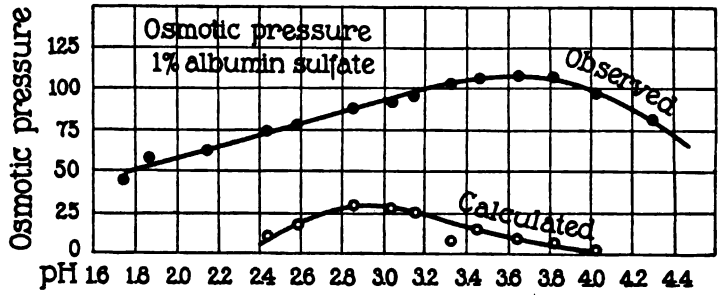

FIG. 6. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent crystalline egg albumin sulfate.

VI. The Depressing Influence of Neutral Salts on the Osmotic Pressure of a Gelatin Chloride Solution.

The question arises whether the membrane equilibrium could also be responsible for the depressing influence of salts on the osmotic pressure of protein solutions of a given pH and concentration of originally isoelectric protein. Although Donnan has shown that such a result is to be expected from his theory, the theory does not include all the facts in the case of gelatin chloride solutions. If on one side of the membrane there be a solution of NaCl, on the other side a colloidal salt NaR, where R is the colloidal ion, the real osmotic pressure (P_o) of the colloidal solution NaR can, according to Donnan,

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be calculated from the observed osmotic pressure (P_1) by the following equation,

$$\frac{P_1}{P_o} = \frac{C_1 + C_2}{C_1 + 2C_2}$$

assuming that gelatin is monovalent, which is improbable, where C_1 is the concentration of NaCl and C_2 that of NaR. If C_1 is small compared with C_2, $P_1 = \frac{1}{2} P_o$; if, however, C_2 is small in comparison with C_1, $P_1 = P_o$. It follows from this that the greater the concentration of NaCl (or of any neutral salt) added to a colloidal solution, the smaller the observed osmotic pressure of the colloidal solution becomes, and this is what actually happens. It follows also from this theory that the observed osmotic pressure cannot be depressed below a certain minimum. This seems to agree with the observation that when the solution of the salt reaches the value M/8 a further increase in the concentration of the salt can no longer increase the depressing effect of the salt (see Figs. 1 and 2 in the preceding paper of this series).[2] To this extent Donnan's theory accounts for the depressing action of the salt on the osmotic pressure. A discrepancy between theory and observed value of osmotic pressure arises, however, in the fact that according to the theory the maximal depression caused by a salt should be $\frac{1}{2}$ the real osmotic pressure of the colloidal solution. The observed osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.5 is about 425 mm. H_2O while the osmotic pressure in the presence of M/8 NaCl is only about 30 mm. According to the theory the latter value should be at least $\frac{1}{2}$ of 425; *i.e.*, 212.

Donnan's theory can only give approximate results in this case since in his theory complicating factors were intentionally ignored for the sake of simplification. Thus the depressing effect of the addition of a neutral salt on the electrolytic dissociation of the electrolyte is not taken into consideration.

Northrop[7] has shown by conductivity measurements that the degree of ionization of gelatin chloride is noticeably depressed when the pH is 2.0 or below and is considerable when the pH is 1.0. There may be other complicating factors besides this depression of ionization.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 211.

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VII. Theoretical Remarks.

It is of interest to compare the colloidal speculations with the almost quantitative results at which we arrived. All colloidal theories would agree in ascribing the effect of the hydrogen ion concentration or of the valency of the ions with which the protein is in combination to a modification in the state of the protein particles, such as hydratation or degree of dispersion. The hydratation theory

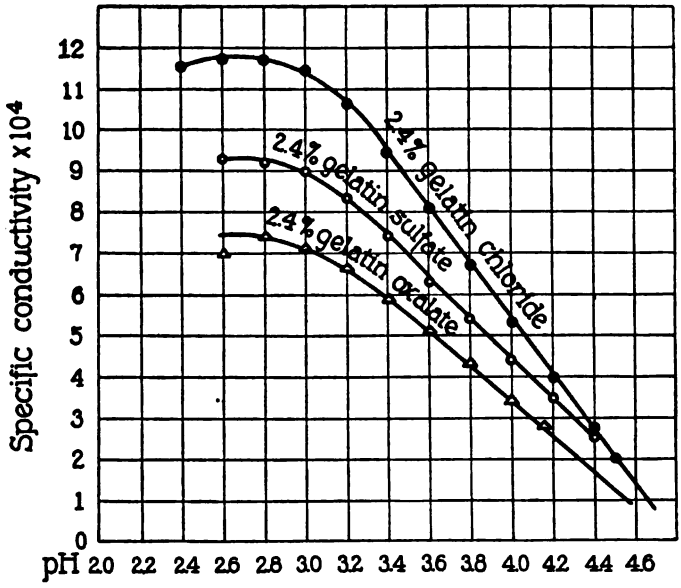

FIG. 7. Curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulfate, and oxalate, showing the entirely different character of these curves from that of the osmotic pressure curves in Figs. 1 and 5 in the preceding paper.

is advocated by Pauli[8] who assumes that the ionized protein particle is strongly hydrated while the non-ionized protein particle is not hydrated. If this were the correct explanation of the peculiar type of curves, such as those reported in Fig. 1 of this paper, we should expect the curves for the specific conductivity of the protein in solution to show a close similarity to the curves in Fig. 1. But such is

[8] Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920.

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not the case. In Fig. 7 are given the curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulfate, and oxalate after deduction of the specific conductivity of the free acid in the gelatin solution, as described in a previous paper. A comparison between Fig. 7 and Fig. 1 fails to show any close similarity. In the conductivity curves there is no maximum followed by a drop at pH 3.5, as there is in the osmotic pressure curves. Fig. 8 shows that the

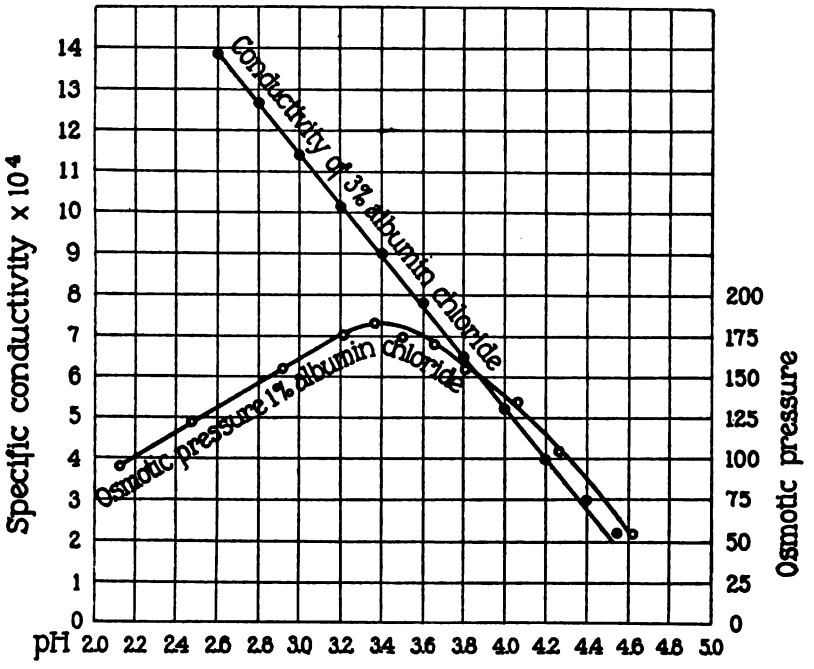

FIG. 8. Comparison of conductivity curve and osmotic pressure curve for albumin chloride, showing the entirely different character of the two curves.

difference between the conductivity curve and the osmotic pressure curve is still more pronounced in the case of albumin chloride.

Manabe and Matula,[9] who claim to have proved Pauli's theory, speak of a maximum of the physical properties of protein solutions at pH 2.0 or 2.1. There seems to be some misunderstanding, since the maximum for osmotic pressure of solutions of protein-acid salts;

[9] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

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e.g., gelatin or crystalline egg albumin, lies at a pH of 3.5 or 3.4, and for casein at a pH above 3.0. The maximal swelling lies at a pH of gelatin of about 3.2 or 3.3. Those who state that it lies at a lower pH (*e.g.* Michaelis[10]) must have mistaken the pH of the supernatant liquid for the pH of the solid gelatin, thus ignoring the effects of the Donnan equilibrium. The maximum for the viscosity of gelatin-acid salts lies at pH of 3.0 and for the viscosity of casein chloride or phosphate at pH 3.0 or above.

Northrop[7] has observed that a drop in the conductivity of gelatin solutions occurs when the pH falls below 2.0, but this cannot explain the drop in the osmotic pressure curves observed at pH above 3.0, and Northrop's results agree entirely with my own in not having noticed a drop in the conductivity curves at pH 3.0 or 3.5.

Moreover, Fig. 7 shows that there is only a slight difference between the conductivity curves for gelatin sulfate and gelatin chloride, while there is a greater difference between the conductivity of gelatin chloride and gelatin oxalate. All this disagrees entirely with the osmotic pressure curves in Fig. 5 in the preceding paper. Furthermore, the idea of a noticeable hydratation of the protein ion seems to be no longer tenable on the basis of Lorenz[11] and Born's[12] experiments and conclusions.

A second colloidal hypothesis would lead us to assume that variations in the degree of dispersion of the protein particles are responsible for the osmotic pressure curves represented in Fig. 1. We need not dwell on this hypothesis since we have no way of putting it to a quantitative test.

The results of this paper show that if we assume the correctness of Donnan's theory of membrane equilibrium the characteristic influences of pH and valency on the osmotic pressure appear as a necessary consequence of the theory; with the exception of the two minor differences discussed in Chapter IV. Donnan's theory leads to a view radically different from all colloidal speculations since on the basis of this theory the variations in osmotic pressure depend on the

[10] Michaelis, L., Praktikum der physikalischen Chemie insbesondere der Kolloidchemie, Berlin, 1921.

[11] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.

[12] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

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unequal distribution of the crystalloidal ions on the opposite sides of the membrane and not on variations in so called colloidal properties of proteins. The quantity which changes with the pH and the valency of the anion of a protein-acid salt is on this assumption not the degree of hydratation or dispersion of the protein particles but the value pH inside minus pH outside, as stated more fully in the two preceding papers.[1]

SUMMARY.

1. It had been shown in previous publications that the osmotic pressure of a 1 per cent solution of a protein-acid salt varies in a characteristic way with the hydrogen ion concentration of the solution, the osmotic pressure having a minimum at the isoelectric point, rising steeply with a decrease in pH until a maximum is reached at pH of 3.4 or 3.5 (in the case of gelatin and crystalline egg albumin), this maximum being followed by a steep drop in the osmotic pressure with a further decrease in the pH of the gelatin or albumin solution. In this paper it is shown that (aside from two minor discrepancies) we can calculate this effect of the pH on the osmotic pressure of a protein-acid salt by assuming that the pH effect is due to that unequal distribution of crystalloidal ions (in particular free acid) on both sides of the membrane which Donnan's theory of membrane equilibrium demands.

2. It had been shown in preceding papers that only the valency but not the nature of the ion (aside from its valency) with which a protein is in combination has any effect upon the osmotic pressure of the solution of the protein; and that the osmotic pressure of a gelatin-acid salt with a monovalent anion (*e.g.* Cl, NO_3, acetate, H_2PO_4, HC_2O_4, etc.) is about twice or perhaps a trifle more than twice as high as the osmotic pressure of gelatin sulfate where the anion is bivalent; assuming that the pH and gelatin concentrations of all the solutions are the same.

It is shown in this paper that we can calculate with a fair degree of accuracy this valency effect on the assumption that it is due to the influence of the valency of the anion of a gelatin-acid salt on that relative distribution of the free acid on both sides of the membrane which Donnan's theory of membrane equilibrium demands.

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3. The curves of the observed values of the osmotic pressure show two constant minor deviations from the curves of the calculated osmotic pressure. One of these deviations consists in the fact that the values of the ascending branch of the calculated curves are lower than the corresponding values in the curves for the observed osmotic pressure, and the other deviation consists in the fact that the drop in the curves of calculated values occurs at a lower pH than the drop in the curves of the observed values.

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THE RÔLE OF THE ACTIVITY COEFFICIENT OF THE HYDROGEN ION IN THE HYDROLYSIS OF GELATIN.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 9, 1921.)

I. The Course of the Reaction.

It is generally assumed that enzymes, and catalysts in general, merely accelerate the velocity of a reaction already taking place. In the case of most enzymes the reaction without the enzyme has been carefully studied and is at least as well known as the enzyme reaction. The hydrolysis of proteins without the presence of enzymes, however, has apparently received very little attention, at least from the standpoint of the kinetics of the reaction. It seemed advisable, therefore, in connection with the study of the proteolytic enzymes to secure some data in regard to the spontaneous hydrolysis, especially since the writer had found that the hydrolysis of gelatin by pepsin[1] was very closely connected with the ionization of the protein. It seemed important to determine whether any influence of the ionization could be noticed in the spontaneous reaction. As will be described more fully below, this is found to be the case.

EXPERIMENTAL.

Preparation of the Gelatin.—Gelatin was selected as the protein in these experiments since it had already been used in the study of pepsin hydrolysis and also since it does not precipitate in strong acid and alkali. It is also much more accurately titratable by the formol titration than are the other proteins. The gelatin was prepared from Cooper's powdered gelatin by washing at the isoelectric point as described by Loeb,[2] and was used in a concentration of

[1] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 211.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

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from 2 to 3 per cent. It was practically salt-free. Several different preparations were made during the course of the work. No difference could be detected in the behavior of these different lots.

The increase in free amino or free carboxyl groups is the most significant quantity as regards the hydrolysis of proteins, since if the accepted views of structure of the proteins are correct, each hydrolytic cleavage results in the liberation of a free amino and a free carboxyl group. Two methods are available for following quantitatively the course of such reaction; Van Slyke's[3] amino nitrogen determination, and Sörensen's[4] formol titration which determines the free carboxyl groups. For absolute determinations of the amino-acids Van Slyke's method is more accurate, for comparative experiments concerning the changes occurring in gelatin solutions, such as were used in this work, the formol titration is more accurate and also much more rapid. Such a slight hydrolysis of gelatin as that required merely to liquefy the protein may be quite accurately determined by the formol titration whereas the increase in amino nitrogen is so small as to be within the limits of error of the Van Slyke method. The formol titration was used therefore in nearly all the experiments reported in this paper. It is well known that many substances, such as phosphates and carbonates, interfere with this titration. In order to avoid these difficulties the method was slightly modified. It is obvious that the final titration figure will depend on the amount of alkali or acid present in the original solution. In order to get comparable results, therefore, it is always necessary to start the titration from the same pH. This was accomplished by titrating the sample to pH 7.0, using neutral red as an indicator, before adding the formaldehyde and continuing with the final titration. In the case of gelatin itself, this method does not give the correct figure since, in order to get the total acidity, it is necessary to start the titration at the isoelectric point of the gelatin; *i.e.*, pH 4.7. The figures obtained by titrating from pH 7.0 are, therefore, too low by the amount of alkali necessary to titrate the gelatin from pH 4.7 to pH 7.0. Since this is a constant quantity for any given concentration of gelatin, the titration figure could be corrected if necessary.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

[4] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

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This correction is small, however, and in the case of comparative experiments cancels out. In the titration of the amino-acids the correction is within the limits of error of the titration. A pH of 7.0 was chosen as the standard since the titration curve of gelatin is very flat at this point so that the adjustment of the reaction is accurate to less than 0.05 cc. of 0.1 N NaOH and also since the presence of citrate does not interfere at this pH.

Adjustment of the pH of Gelatin Solutions.—In strongly acid or alkaline solutions it is not necessary to use buffers since the change in the pH during the hydrolysis of the gelatin is neglible. Between pH 1.5 and 11.0, however, it is necessary to use some buffer as otherwise the pH of the solution changes rapidly during the course of the reaction. It was noted above that phosphates and carbonates interfere with the titration. Phosphates are difficult to remove and were not used at all in these experiments. It was found that a complete series of buffer solutions could be prepared with various mixtures of trisodium citrate, HCl, and Na_2CO_3. These solutions were therefore used. Since carbonate also interferes with the titration it was removed by bringing the sample to about pH 2.5 with strong acid, boiling out the CO_2 and then titrating back to pH 7.0. Control experiments showed that this procedure had no effect whatever on the final titration; that is, the figure obtained for 10 cc. of gelatin solution was identical when the gelatin was titrated to pH 7.0 directly before making the formol titration, and when it was titrated first to say pH 10 with Na_2CO_3, then made acid, the carbonate boiled out, and then titrated to pH 7.0. The total concentration of citrate was not above 0.05 N, since more concentrated solutions caused a slight increase in the titration figure and rendered the adjustment to pH 7.0 more difficult.

Formalin Solution.—A solution of formalin was used containing 30 cc. of a saturated aqueous solution of thymol blue per 300 cc. of solution, and titrated with sufficient alkali, so that when 5 cc. were added to 15 cc. of water, the resulting solution had a pH of 8.4. This procedure obviates the necessity of making a correction for the formalin solution. 5 cc. of this solution were used for 10 cc. of the sample.

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Example of Titration.—(*a*) *Solution Containing no Carbonate.*—10 cc. of 2.5 per cent gelatin solution or the equivalent amount, if more concentrated gelatin is used, are pipetted into a large test-tube, 1 drop of neutral red is added, and the solution is brought to approximately pH 7.0 by the addition of a few drops of concentrated alkali (carbonate-free) or strong HCl. The reaction is then corrected accurately to pH 7.0 with 0.1 N NaOH (CO_2-free) or 0.1 N HCl, using a standard of pH 7.0 in the comparator block. 5 cc. of the formalin solution are then added and the solution is titrated with 0.1 N NaOH to pH 8.4, using a standard tube with thymol blue for comparison. The neutral red does not interfere with this titration and if anything makes it more sharp. The number of cc. of 0.1 N alkali required to titrate from pH 7.0 to pH 8.4 after the addition of the formalin is noted and is referred to as the formol titration. This value, as Sörensen showed, is independent of the final pH taken, provided this is above 8.2. It represents the number of cc. of 0.1 N acid present in the solution.

(*b*) *Carbonate Present.*—The solution is brought to pH 2.5–3.0 with a few drops of concentrated HCl and boiled for 10 to 20 seconds over the free flame to remove the CO_2. It is then titrated as described under (*a*).

In order to check the accuracy of the method several experiments were made in which the results obtained by the formol titration and by Van Slyke's method were compared. The results of a few of these are given in Table I. The amino nitrogen was determined as described by Van Slyke. The solution was shaken for 30 minutes. In the case of gelatin, the titration figures for gelatin alone, without the addition of formalin are also given. These figures were obtained from the titration curves of gelatin given by Loeb,[5] by correcting for the amount of acid or alkali necessary to bring the same volume of water to the same pH. It is found that below pH 1.8 and above pH 11.0 at 25°C. the figures so obtained are constant and independent of the pH; *i.e.*, outside these limits, the gelatin is all combined as a salt and the figures represent the normality of the solution.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85.

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TABLE I.

Analysis of Gelatin Solutions by Different Methods.

Solution analyzed.	NH_2N per 2 cc. of solution, Van Slyke method.	Formol titration per 10 cc. of solution.	Equivalent cc. of 0.1 N NaOH calculated to 1 cc. 1 per cent gelatin.		Normality 1 per cent gelatin.
			Van Slyke.	Formol.	
	cc.	*cc.*	*cc.*	*cc.*	
5 per cent gelatin.	0.94	3.00 (direct titration.)	0.036	0.060	0.0036 (Van Slyke).
	0.88	3.05	0.038	0.061	0.0038
		2.05 (titration to pH 7.0. first; neutral red titration.)		0.040	0.0060 (Formol).
		2.03		0.041	
5 per cent gelatin slightly hydrolyzed (just liquefied).	1.00	2.30 "	0.040	0.046	
	1.05	2.25 "		0.045	
2 per cent gelatin partially hydrolyzed.	5.00	11.60 "	0.60	0.58	
	4.80	11.50 "	0.57	0.575	
2.5 per cent gelatin nearly completely hydrolyzed at pH 14.0 containing 0.1 N Na_2CO_3.	10.0	19.5 "	0.80	0.83	
	10.4	20.8 "	0.84	0.78	
1 per cent gelatin titrated directly with HCl or any strong acid.			0.120		0.012
1 per cent gelatin titrated directly with NaOH or Ba(OH).			0.06		0.006

The table shows that the formol titration, if the gelatin is first brought to pH 7.0, gives identical figures with that of the Van Slyke determination at all stages of hydrolysis. It also shows that the direct titration of gelatin without any formalin gives the same value

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as that obtained with the formol titration. The formaldehyde evidently merely makes the acid groups stronger so that it is not necessary to titrate to such a high pH.

The figures in the last column show that 1 per cent gelatin solution is about 0.004 normal with respect to free NH_2 groups, 0.006 normal with respect to total acid groups, and 0.012 normal with respect to total basic groups. That is, there must be approximately twice as many basic as carboxyl groups, and three times as many total basic groups as NH_2 groups. These figures agree approximately with those found by Wintgen and Krüger.[6]

Determination of Hydrogen Ion Concentration.—The pH of the solutions was determined before and after hydrolysis. In the case of the buffered solutions no significant change was noted. The determination was made by the E.M.F. method (except in the solutions containing $HgCl_2$) using the rocking electrodes as described by Clark[7] in a constant temperature bath. A saturated KCl calomel electrode was used. The E.M.F. of this electrode was determined before and after every series of determinations against a 1.0 N solution of hydrochloric acid, prepared according to Hulett[8] and checked by conductivity measurements. The pH of this 1.0 N HCl was calculated from the activity coefficient as given by Noyes and MacInnes;[9] *i. e.*, 0.082 at 25°C. This standard was chosen since Fales and Vosburgh[10] have shown that there is no diffusion potential in such a cell and since the strength of the acid can be checked by an independent method; *i.e.*, the conductivity. The value for the saturated KCl electrode found in this way averaged 244 millivolts. It varied about 1 millivolt on either side of this value, from time to time, but was constant within 1 millivolt during the course of any one series of determinations.

Influence of Temperature on the pH.—Since the experiments were carried out at 40°C. and most of the pH determinations were made at 25°C. it was necessary to know what effect this change of tempera-

[6] Wintgen, R., and Krüger, K., *Koll. Z.*, 1921, xxviii, 81.
[7] Clark, W. M., Determination of hydrogen ions, Baltimore, 1920.
[8] Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 390.
[9] Noyes, A. A., and MacInnes, P. A., *J. Am. Chem. Soc.*, 1920, xlii, 239.
[10] Fales, H. A., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1918, xl, 1291.

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ture would have on the pH. In order to determine the value of this temperature effect a series of gelatin solutions containing 2 per cent gelatin and having a pH of from 0.0 to 14.0 was made up. The pH was adjusted by varying amounts of HCl, NaOH, Na_2CO_3, and sodium citrate. The total salt content of all the solutions was between 0.02 and 0.05 N. The pH of these solutions was then determined at 25° and at 37°C. (The electrodes regulated at 37°C. were put at the author's disposal by Dr. Glenn E. Cullen). The results are summarized in Table II. The C_{OH} was calculated by the formula $C_H C_{OH} = K_W$ (or pH + pOH = Log K_W). The value of K_W at 25° was taken as 1×10^{-14} and at 40° as 2.8×10^{-14}. It was found that the hydrogen ion concentration was independent of the tem-

TABLE II.

Effect of Temperature on pH of Gelatin Solution.

pH observed.		pOH calculated.	
25°C.	40°C.	25°C.	40°C.
12.70	12.25	1.30	1.25
12.25	11.75	1.75	1.75
9.92	9.72	4.08	3.78
8.42	8.42	5.58	5.08
5.01	5.02	8.99	8.48
0.76	0.75	13.24	12.75

perature from pH 0.0 to pH 8.8, and that the (calculated) hydroxyl ion concentration was independent of the temperature from pH 11 to pH 14. Between pH 8.8 and 11.0 the concentrations of both hydrogen and hydroxyl ions increased with the temperature. In all the other experiments therefore the hydrogen ion concentration was assumed to remain the same at 25° and 40°C. on the acid side of pH 8.5, and the hydroxyl ion concentration was assumed to remain constant on the alkaline side of pH 11.0. The experiments which fell between pH 8.8 and 11.0 were measured at 37°C.

The Course of the Reaction.—It has been known for a long time that proteins may be hydrolyzed to the amino-acids by prolonged heating with acids or alkali. The kinetics of the reaction, however, have received little or no attention. Since it was desired to compare the

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velocity of hydrolysis of the gelatin under different conditions it was first necessary to determine some value to be used as a standard. Two gelatin solutions were therefore prepared, one containing 0.75 N NaOH and the other 4.5 N HCl. These solutions were then placed at 65°C. and the increase in the formol titration followed. The results are shown in Fig. 1. The curves fall off more rapidly than the predicted rate of a monomolecular reaction, as is shown by Fig. 2, in which the logarithm of the quantity of gelatin remaining is plotted against the time, which in the case of a monomolecular

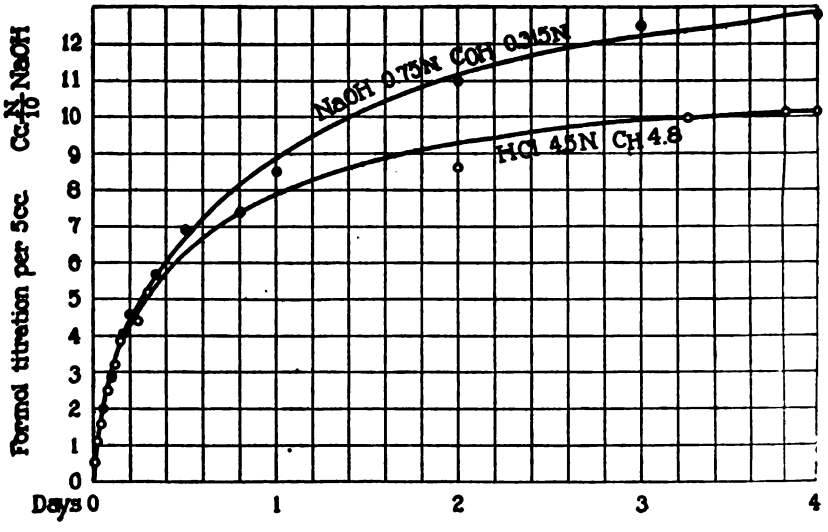

FIG. 1. Increase, with time, in formol titration in strongly alkaline and strongly acid solutions.

reaction gives a straight line. It is obvious from the figure that the reaction is monomolecular for the first 30 or 40 per cent but than becomes too slow. This cannot be ascribed to changes in the C_H or C_{OH} since no measureable change in these values could be detected. This means presumably that the simpler decomposition products are more resistant to hydrolysis than is the gelatin itself. Schroeder[11]

[11] von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 75. Taylor (Taylor, A. E., *Univ. California Pub., Path.*, 1907, i, 239) states that the hydrolysis of protamine by acid is monomolecular.

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found that the rate of decrease of the viscosity of gelatin solutions also follows the monomolecular formula. This would be expected since the decomposition products have a very low viscosity compared to gelatin. It may be mentioned that the final figure reached by the two solutions was nearly identical and agreed with that found by Van Slyke for the total hydrolysis of gelatin. The alkaline solution gave a slightly higher figure due to silicic acid from the glass. Since the gelatin must evidently be destroyed in the very beginning of the reaction, it is this part which must be studied in order to notice any influence of the condition of the gelatin on the velocity of hydrolysis. It will be noticed that the formol titration increases about

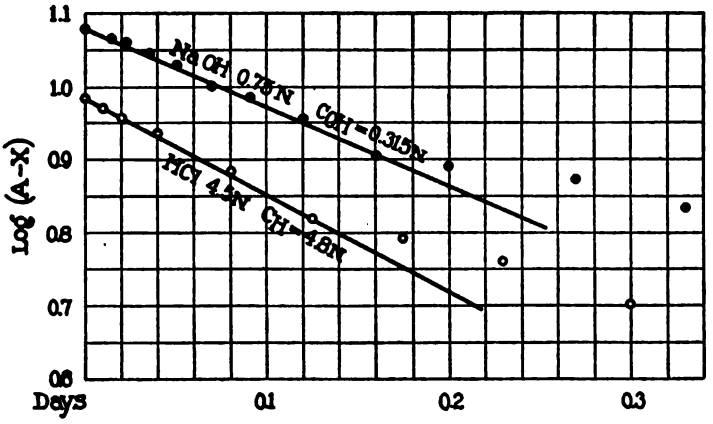

FIG. 2. Increase, with time, in formol titration in strongly alkaline and strongly acid solutions.

20 times during the reaction so that during the first two or three hundred per cent increase in the original figure the increase will be very nearly linear with respect to time.

That this is actually the case is shown in Fig. 3 in which the increase in the formol titration, as expressed in per cent of the original figure is plotted against the time. As the figure shows, the curves of all the experiments in which the hydrogen ion concentration was kept constant are straight lines within the limits of experimental error. Curve 4 in which the hydrogen ion concentration decreased during the hydrolysis, however, drops off quite rapidly. As will be shown

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later, the velocity in this range of pH is proportional to the hydrogen ion concentration. Also since the products formed are weak bases we have the condition that[12]

$$R\mathrm{NH_2} + \mathrm{HCl} \rightleftharpoons R\,\mathrm{NH_2.HCl} \qquad (1)$$

and hence, when $R\mathrm{NH_2}$ is large compared to C_H; (assuming complete ionization of the HCl)

$$C_H = \frac{k}{C_{R\mathrm{NH_2}}}$$

i.e., the hydrogen ion concentration and hence the velocity of the reaction will be inversely proportional to the amount of products formed.

$$\text{Velocity} = \frac{dx}{dt} = k\,C_H = \frac{k'}{C_{R\mathrm{NH_2}}} = \frac{k'}{x}$$

This, as Arrhenius[13] pointed out is the condition which causes Schütz's rule since on integration it becomes

$$x = k'' \sqrt{t}$$

In Fig. 4 the results have been plotted against the square root of the time and it will be seen that the experiment in which the hydrogen ion concentration was *not* kept constant (by means of buffers) obeys Schütz's rule very well; *i.e.*, the amount of products formed is proportional to the square root of the time. Reactions in which the rate is inversely proportional to the amount of products formed have been quite frequently observed. A very clear case which was completely worked out and the mechanism verified experimentally is that of the oxidation of oxalic acid by bromine studied by Richards and Stull.[14] Similar reactions are discussed by Müller.[15] The writer

[12] The complete equation would be

$$\begin{array}{l} \mathrm{RNH_2} \\ \end{array} \begin{array}{l} + \mathrm{HCl} \rightleftharpoons R\mathrm{NH_2} \\ + \mathrm{H_2O} \rightleftharpoons R\mathrm{NH_2OH} \end{array} \begin{array}{l} . \ \mathrm{HCl} \rightleftharpoons R\mathrm{NH_3^+} + \mathrm{Cl^-} \\ \downarrow\uparrow \\ R\mathrm{NH_2^-} + \mathrm{H^+} \end{array}$$

At the range of pH under consideration, however, the equilibrium may be represented approximately by (1).

[13] Arrhenius, S., *Medd. Kong. vetsakad. Nobelinst.*, 1908, i.

[14] Richards, T. W., and Stull, W. N., *Z. physik. Chem.*, 1902, xli, 544.

[15] Müller, W., *Z. physik. Chem.*, 1902, xli, 483.

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was able to show[16] that in pepsin digestion the same mechanism causes the reaction to follow the square root law since the pepsin and the products of digestion are in equilibrium. The pepsin in this case replaces the hydrogen ion in the present example.

Returning to the experiments in which the hydrogen ion concentration was kept constant it will be seen that the rate of reaction is practically constant for at least the first 400 per cent increase in the original titration (equivalent to the first 20 per cent of the reac-

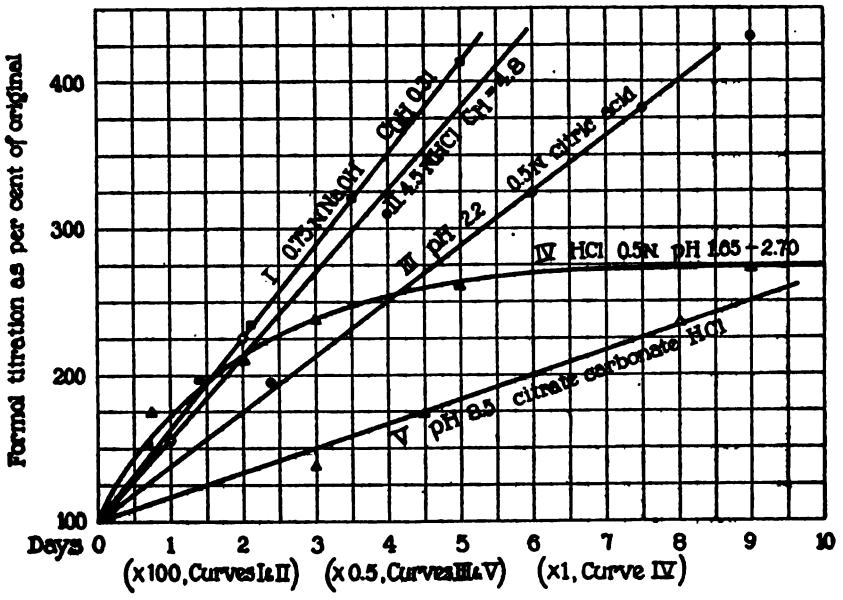

FIG. 3. Course of reaction with various hydrogen ion concentrations.

tion). The velocity within this range may, therefore, be determined at any time by dividing the increase in the titration by the elapsed time. If the increase is expressed as per cent of the original figure this ratio will represent the velocity of the reaction and will be independent of the concentration of gelatin. It seems more convenient and equally significant for the purposes of this paper to use this figure rather than the constant for the monomolecular reac-

[16] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

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tion. The velocity will therefore be considered as the increase expressed as per cent of the original titration divided by the elapsed time in days. The resulting figure is the per cent increase in the original titration per day, assuming that the amount of unhydrolyzed gelatin remains approximately unchanged. In most of the experiments determinations were made at several different times and the velocities so obtained were averaged. In all cases the experiment

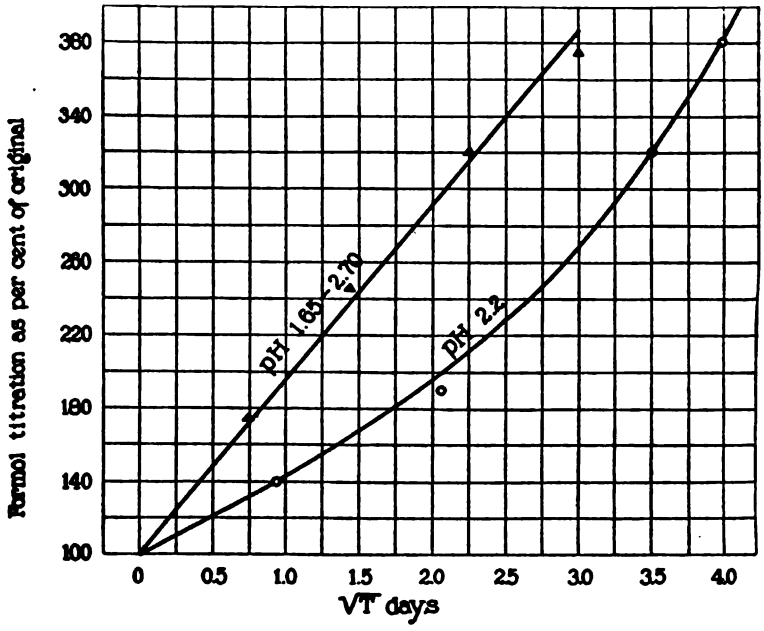

FIG. 4. Comparison of the course of reaction with constant and with varying hydrogen ion concentrations.

was continued until the original titration had increased at least 50 per cent and in all, except a few between pH 5.0 and 7.0, until the titration value had increased over 200 per cent.

Concentration of Gelatin.—Since the reaction approximates that of a monomolecular, the velocity when expressed as percentage change should be independent of the gelatin concentration. Table III contains the result of an experiment in which the gelatin concentration was varied from 10 to 1.25 per cent; it shows that the velocity is independent of the concentration for the early stages of the hydrolysis.

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TABLE III.

Influence of Concentration of Gelatin at 65°C.
Gelatin Per Cent Concentration Noted + HCl to pH 2.0.

Concentration.	C_H	Velocity = $\frac{\text{per cent change}}{\text{time in days}}$.
per cent		
10	2.00	16
5	2.08	16, 14
2.5	2.03	17
1.25	2.02	17, 18

II. The Influence of High Concentrations of Hydrogen and Hydroxyl Ions.

It was shown above that the rate of the reaction decreased very rapidly if the hydrogen ion concentration was allowed to decrease. This in itself indicates that the rate is a function of the hydrogen ion concentration and not of the total amount of acid present. The fact that it is the hydrogen ion concentration and not the total acidity

TABLE IV.

Influence of Different Acids on Velocity of Hydrolysis.
2.5 Per Cent Gelatin at 65°C. with pH Measured at 25°C.

Acid.	Concentration.	C_H 25° × 10^9.	Velocity.	$\frac{\text{Velocity}}{C_H} \times 10^{-8}$.
	N			
HCl	0.024	6.0	18	3.0
HNO_3	0.024	6.0	17	2.8
H_2SO_4	0.024	6.3	16	2.5
Oxalic	0.050	6.3	16	2.5
Citric	0.33	5.6	16	2.8
		5.1	15	2.9

is shown more clearly in Table IV which contains the results of an experiment in which the gelatin was brought to the same pH with different acids. The table shows that the velocity is the same in all the solutions although the total concentration of citric acid was more than 10 times that of the HCl.

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In all the above experiments the hydrogen ion concentration has been expressed as that calculated by Nernst's formula from the potential of the hydrogen electrode. It is certain that this value does not really express the actual concentration in grams per liter of hydrogen ions. It would probably be better to call it the "activity coefficient" or "active concentration" as proposed by Lewis;[17] that is, it represents that value which must be assigned to the hydrogen ion concentration in order to have it obey the law of mass action as assumed in Nernst's equation. In low concentration, however, the value so obtained does not differ very much from that obtained by the conductivity method. In more concentrated solution. the discrepancy becomes greater and may amount to several hundred per cent in concentrated HCl. It seemed of interest to determine which of these values is the significant one for the hydrolysis of gelatin. A series of experiments was therefore made in which the concentration of acid was varied from 1.0 N to about 4.1 N acid. The results are shown in Table V. The total acidity was determined approximately by titration. The values for the hydrogen ion concentration by the conductivity measurements were interpolated from Kohlrausch's tables. The hydrogen ion concentrations as determined by the E. M. F. method agree fairly well with those given by Noyes and MacInnes[9] except in the case of the strongest HCl concentration in which it is too low. It is remarkable that the rate of hydrolysis is in all cases nearly proportional to the hydrogen ion concentration as determined from the E. M. F. measurements. It is clear therefore that it cannot be the concentration of hydrogen ions as expressed in grams per liter which determines the rate of the reaction, since in the case of 3.7 N HCl it would be necessary to assume nearly 120 per cent dissociation. On the other hand, the result cannot be ascribed to the undissociated acid since it is hardly conceivable that the undissociated acid could effect the hydrogen electrode. It seems necessary to conclude that it is the "activity" of the hydrogen ion which determines the rate of hydrolysis of gelatin as well as the potential of the hydrogen electrode.

[17] Lewis, G. N., *Proc. Am. Acad. Arts and Sc.*, 1907, xliii, 259.

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In other words, the "concentration" as determined by the hydrogen electrode is the effective concentration for the present reaction.

In a very recent paper Schreiner[18] has suggested the use of a special "catalysis-coefficient" to express the relation of the apparent hydrogen ion concentration, as determined by catalysis experiments, to the total acid concentration. Schreiner states that this coefficient is equal to the reciprocal of the conductivity ratio (corrected for

TABLE V.

Hydrolysis of 2.5 Per Cent Gelatin in Strong Acid at 40°C.

Acid.	Approximate concentration.	C_H E.M.F.	C_H Conductivity.	Velocity.	$K_H = \frac{\text{Velocity}}{C_H \text{ (E.M.F.)}}$	$\frac{\text{Velocity}}{C_H \text{ (conductivity)}}$
	M					
HCl.....	4.1	[5.5]	1.97	800	[130]	400
HCl.....	3.7	5.4	1.88	790	146	400
H_2SO_4.....	3.7	2.0	1.57	300	151	200
HCl.....	3.1	3.6	1.75	600	167	340
H_2SO_4.....	3.0	1.6	1.40	180	[125]	130
HCl.....	2.2	2.7	1.48	380	140	250
HCl.....	2.1	2.10	1.40	[400]	[190]	[280]
H_2SO_4.....	2.0	0.90	1.00	130	146	130
HCl.....	1.5	1.50	1.06	220	140	210
HCl.....	1.0	0.84	0.80	142	170	178
HCl.....	1.0	0.85	0.80	130	160	162
HCl.....	1.0	0.89	0.80	[160]	[180]	[200]
H_2SO_4.....	1.0	0.40	0.50	60	150	120
HCl.....	0.7	0.72	0.60	110	155	185
K_H = average.....					152	

viscosity), and he is able by its use to calculate quite closely the velocity of hydrolysis of methyl and ethyl acetate in concentrated acid or acid-salt mixtures. It seems unnecessary to introduce such a coefficient in order to account for the results of the experiments reported here, since it is possible to calculate the velocity, within the rather large experimental error, by means of the activity coefficient alone.

[18] Schreiner, E., *Z. anorg. u. allg. Chem.*, 1921, cxvi, 102.

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III. Effect of Neutral Salts.

It was noted by Arrhenius[19] in 1899 that the addition of neutral salts to a weak acid increased the rate of hydrolysis of methyl acetate by the acid, which is just the opposite of the effect expected from the law of mass action. Arrhenius originally assumed that the salt actually increased the concentration of hydrogen ions. This view was criticised, however, and the hypothesis was put forward independently[20] by Dawson, and by Senter and Acree that the observed effect was due to the undissociated acid and a large number of experiments were performed which strengthened this view. It was found by Nelson and Fales,[21] however, that under certain conditions the effect of neutral salts on the activity of invertase could be ascribed entirely to the effect of the salt on the hydrogen ion concentration as measured by the hydrogen electrode. Since that time evidence has accumulated to show that the addition of neutral salts to a strong acid causes an increase in the hydrogen ion concentration as measured by the hydrogen electrode. A series of experiments was therefore performed to determine whether the addition of salts would influence the hydrolysis of gelatin. The results of this series are given in Table VI. It will be seen that the addition of 1.5 N NaCl to 1.0 N HCl solution increases the hydrogen ion concentration by nearly 50 per cent and that the rate of hydrolysis is increased practically the same amount. That is, if the effect of the neutral salt on the hydrogen ion concentration is taken into account, there is no effect on the rate of hydrolysis. In the case of NaCl and $CaCl_2$ the result is the same up to 1.0 N but above that the hydrogen ion concentration is increased more than the rate of hydrolysis. It was thought at first that this might be due to diffusion potentials caused by the NaCl or $CaCl_2$, since it will be noted that NaCl and $CaCl_2$ increase the hydrogen ion concentration more than the same concentration of KCl, whereas the effect on the rate of hydrolysis is the same for all the salts. (This is the result obtained

[19] Arrhenius, S., *Z. physik. Chem.*, 1899, xxxi, 197.

[20] For a review of this question see Lewis, W. C. McC., A system of physical chemistry, London, 1918–19, i.

[21] Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2769.

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TABLE VI.

Effect of Addition of Salts on Hydrolysis of 2.5 Per Cent Gelatin at 40°C.
HCl Concentration 1.0 N.

Salt.	Concentration.	pH	C_H E.M.F.	Velocity.	$K_H = \frac{\text{Velocity.}}{C_H}$
	N				
0	0	0.07	0.85	142	168
0	0	0.08	0.83	140	169
KCl	1.0	+0.06	1.15	176	153
KCl	1.0	+0.05	1.12	177	158
KCl	1.5	+0.08	1.20	182	151
KCl	1.5	+0.08	1.20	185	154
0	0	0.07	0.84	145	172
NaCl	0.5	+0.17	1.04	170	163
NaCl	1.0	+0.09	1.23	180 190	146 155
NaCl	1.5	+0.17	1.48	180	120
NaCl	1.65	+0.20	1.58	187	118
NaCl	1.65	+0.20	1.58	172	109
NaCl	1.65	+0.24	1.74	174	100
$CaCl_2$	0.5	+0.034	1.08	162	150
$CaCl_2$	1.0	+0.08	1.20	178	148
$CaCl_2$	1.5	+0.23	1.70	185	110
65°C. HCl Concentration 0.02 N.					
0	0		0.0049	10	2.0×10^3
0	0		0.0045	11	2.4×10^3
KCl	0.5		0.0043	10	2.3×10^3
KCl	0.5		0.0043	13	3.0×10^3
KCl	1.0		0.0043	12	2.8×10^3
KCl	1.0		0.0045	10	2.2×10^3
KCl	1.5		0.0041	12	2.9×10^3
KCl	1.5		0.0041	12	2.9×10^3

by Arrhenius also.) Measurement showed, however, that there was no diffusion potential in the case of NaCl at least. The diffusion potential was measured by determining the E. M. F. of the hydrogen electrode immersed in the NaCl-HCl solution, first against the sat-

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urated KCl calomel electrode (in which case the hydrogen electrode in the NaCl-HCl is negative), and second, against another hydrogen electrode immersed in 1.0 N acid; a saturated KCl bridge was used in both cases. In this case the electrode in the NaCl solution is positive. Any diffusion potential therefore would tend to increase the measured potential in one case and decrease it in the other, so that the potential between the two hydrogen electrodes when measured directly should differ from the difference in potential of the two measured against the same calomel electrode by twice the value of the diffusion potential. The experiment showed, however, that an identical value was obtained by either method.

The experiments summarized in Table VI show that the effect of neutral salts can be accounted for by the increase of the hydrogen ion concentration. Whatever discrepancy is found is due to the fact that the hydrogen ion concentration apparently increased more than the rate of hydrolysis. It is evidently unnecessary to assume any activity of the unionized acid in this case at least.

Experiments in Low Concentration of Acid.—The above experiments were repeated with 0.02 N HCl (at 65°C.). In this case the addition of KCl has a very slight depressing effect on the hydrogen ion concentration and little if any influence on the rate of hydrolysis. This experiment also shows that the unionized gelatin chloride must hydrolyze at approximately the same rate as the ionized. It was shown by conductivity measurements[1] that gelatin chloride is practically completely ionized at pH 2.4, and that the addition of 1.0 N Cl ion reduces the ionization to a very small amount. It also decreases the rate of hydrolysis by pepsin very markedly and in direct proportion to the decrease in the conductivity of the gelatin chloride. The hydrolysis of gelatin by acid and by pepsin differs markedly in this respect. In pepsin hydrolysis the rate of digestion is decreased by increasing the amount of acid or salt beyond 0.01 N whereas in the acid hydrolysis the rate is unaffected by the addition of salt and increases in direct proportion to the hydrogen ion concentration with the addition of more acid. These facts agree with the hypothesis that the rate of hydrolysis of the ionized and non-ionized gelatin salt by hydrogen ions is the same, but that the ionized gelatin salt hydrolyzes much more rapidly than the non-ionized in the presence of pepsin.

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IV. Influence of the Hydroxyl Ion Concentration.

It has been shown above that the velocity of hydrolysis of gelatin is directly proportional to the hydrogen ion concentration as measured

TABLE VII.

Hydrolysis of 2.5 Per Cent Gelatin in Strong Alkali at 40°C.

Alkali.	Concentration.	$C_{OH} = \frac{10^{-13.5}}{C_H}$.	Velocity.	$K_{OH} = \frac{\text{Velocity}}{C_{OH}\ (\text{E.M.F.})\ 25°C.}$
	N			
$Ba(OH)_2$.....	0.16	0.05	250	5,000
KOH.....	0.140	0.047	225	4,800
NaOH.....	0.12	0.045	230	5,100
KOH.....	0.69	0.30	1,000	3,300
NaOH.....	0.78	0.30	1,300	4,330
NaOH.....	1.0	0.45	2,250	5,000
KOH.....	1.27	0.60	2,300	3,900
NaOH.....	1.36	0.63	3,300	5,300
KOH.....	1.91	1.25	5,000	4,000
NaOH.....	2.0	0.90	6,000	6,600
NaOH.....	2.04	0.91	[8,000 9,000]	[9,000 10,000]
NaOH.....	2.72	1.20	10,000	[8,400 7,100]
NaOH.....	3.0	1.26	9,000	
KOH.....	2.55	2.0	8,500	4,250
KOH.....	3.82	3.2	17,000	5.300
NaOH.....	4.08	1.78	20,000	[11,000 7,500]
NaOH.....	4.1	1.86	14,000	
NaOH.....	4.9	2.70	21,000	7,800
Average.....				4,700

by the hydrogen electrode in acid concentrations of from 0.10 to 4.1 N. In Table VII the results of a series of determinations in strong alkali are given. The concentration of hydroxyl ions was calculated

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from the pH, taking the value for the dissociation constant of water at 40°C. as 2.8×10^{-14}. The rate is directly proportional in this range to the hydroxyl ion concentration as determined by the hydrogen electrode. It is not possible to compare the hydroxyl ion concentration as determined by the conductivity since the alkali solutions contained some carbonate. The constant is not so good here as in the acid solution since the reaction in strong alkali is extremely rapid. It will be noticed that the proportionality factor for the hydroxyl ion is 4700 whereas that for the hydrogen ion was 150; *i.e.*, the hydroxyl ions hydrolyze about 30 times more rapidly than hydrogen ions at the same concentration. It is not possible to say whether this difference is due to the hydrogen and hydroxyl ions or to the difference in the ease of hydrolysis of the gelatin-acid salt and the alkali gelatin.

V. Influence of Hydrogen and Hydroxyl Ions in Low Concentration.

It has been shown above that the velocity of hydrolysis in the acid range may be calculated from the equation

$$\text{Velocity}_{H} = 150\, C_{H}$$

and in strongly alkaline solutions from the equation

$$\text{Velocity}_{OH} = 4700\, C_{OH}$$

Combining these two equations we would calculate that (in round numbers) the velocity at any $C_H = 150\ (C_H + 30\ C_{OH})$. This is the same type of equation found by Wijs[22] to represent the influence of the concentration of hydrogen and hydroxyl ions on the rate of hydrolysis of methyl acetate. The formula predicts that the rate of hydrolysis is a minimum when the $C_H = 30\ C_{OH}$ or at 40°C. at about pH 6.0. (The mathematical proof of this statement is that the first derivative of the above expression is zero at the point where $C_H = 30\ C_{OH}$, and would be equal to approximately 3×10^{-4}; *i.e*, it would take nearly 30 years to cause an increase of 3 per cent in the formol titration of a gelatin solution at 40°C.) It was found, however, that the hydrolysis actually occurred at pH 6.0, about

[22] Wijs, J. J. A., *Z. physik. Chem.*, 1893, xii, 514.

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300 times more rapidly than that predicted by the formula. The results of a series of experiments covering the range of from pH 1.0 to pH 12 are given in Table VIII and Fig. 5. In this figure the black circles are the experimental points; the line, the graph of the expression, Velocity = 150 (C_H + 30 C_{OH}); and the circles, the rate calculated from a formula considered below. It is necessary in this range to use some precaution to prevent the growth of microorganisms. Several different substances were used as indicated in the table. Control experiments were made at 65°C. on the influence of these

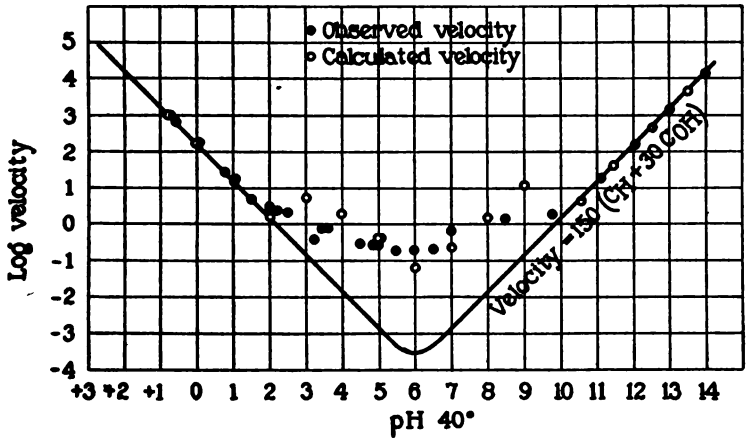

FIG. 5. Influence of the hydrogen ion concentration on the velocity of hydrolysis at 40°C.

on the rate of reaction—no significant effect was noted. In the case of $HgCl_2$ the pH was determined electrometrically before adding the $HgCl_2$ and then colorimetrically. It is a curious fact that nearly all disinfectants poison the hydrogen electrode. The experiment shows that the velocity of hydrolysis is directly proportional to the hydrogen ion concentration from pH 0.65 to pH 2.0. It then decreases much more slowly than the hydrogen ion concentration, passes through a very flat minimum at about pH 6.0 and then increases to become directly proportional to the hydroxyl ion concentration at about pH 10.0. The position of the minimum cannot be told with certainty from the figures given, since the difference

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TABLE VIII.

Buffer.	Preservative.	40°C.	40°C.	Velocity.	$K_1 = \frac{\text{Velocity}}{C_H + 30\,C_{OH}}$.	$K_1 = \frac{\text{Velocity}}{(C_H + 30\,C_{OH})\,(C_{uncombined} + 200\,C_{combined})}$.
		pH	C_H			
See Table V.....		+0.74	5.5 to 1.0	800–100	130–150	130–150
HCl.....		0	1	165	165	165
HCl.....		0.76	0.17	28	165	165
HCl.....		1.0	0.10	15	150	150
HCl.....		1.57	0.03	5	166	160
HCl.....	Toluene	1.80–1.9	1.2 / 1.6 $\times 10^{-2}$	3.0	180 / 250	180
Citric.....	Toluene	2.20	6.3×10^{-3}	2.8	440	350
HCl citrate.....	Benzoate	2.54	2.9×10^{-3}	2.4	830	30
Citrate-HCl.....	$HgCl_2$	3.17	6.8×10^{-4}	0.4	600	150
Citrate-HCl.....	Benzoate	3.60	2.5×10^{-4}	0.8	3,200	15
HCl citrate.....	$HgCl_2$	4.50	3.1×10^{-5}	0.3	10,000	60
	$HgCl_2$	4.90	1.2×10^{-5}	0.3	21,000	120
Citrate HCl.....	$HgCl_2$	4.90	1.2×10^{-5}	0.3	26,000	120
HCl.....	Toluene	4.9	1.2×10^{-5}	0.3	25,000	
	Toluene	5.01	1×10^{-5}	0.5	50,000	225
Citrate.....	$HgCl_2$	5.4	4×10^{-6}	0.1 / 0.2	31,200	150
Citrate.....	$HgCl_2$	6.0	1×10^{-6}		100,000	700
Citrate.....	$HgCl_2$	6.5	3.1×10^{-7}	0.2	64,000	450
			C_{OH} 40°C.			
Citrate-Na_2CO_3....	Benzoate	7.2	5×10^{-7}	0.2	12,000	90
	Benzoate	8.7–8.4	$1.6\text{–}0.8 \times 10^{-6}$	1.8	3,700 / 1,850	150 / 75
Na_2CO_3.....	Thymol Toluene	9.75	1.8×10^{-4}	2.0	370	
NaOH, Na_2CO_3...		11.1	4×10^{-3}	18	150	150
NaOH, Na_2CO_3....	Benzoate	11.1	4×10^{-3}	20	165	165
NaOH.....		12.20	5×10^{-2}	250	165	165
NaOH.....		12.50	0.10	330	110	110
See Table VII....			0.05–3.2	250–1,700	160 ± 10	160

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in the rates is not much greater than the experimental error. The determination of the minimum was checked, however, by noting the degree of liquefaction of the solutions after 90 days at 40°C. 10 cc. of the various solutions were brought to pH 5.0, the salt concentration and total volume made the same in all the tubes, and the tubes then immersed in a water bath at 5° for 2 hours. The degree of liquefaction was then noted. The result is given in Table IX. According to this determination the minimum point is about pH 6.5.

Two hypotheses may be suggested to explain this anomalous influence of low concentrations of hydrogen and hydroxyl ions. First, the velocity of hydrolysis is independent of the hydrogen ion concentration between pH 2.0 and 11.0 (or is proportional to the product of $C_H \times C_{OH}$). Second, some change takes place in the gelatin solution

TABLE IX.

pH	7.2	6.5	6.0	5.3	4.8
Liquefaction after 2 hours, 5°C.	+	±	+	+++	++++

in this range which causes the gelatin to hydrolyze very much more rapidly and so compensate for the decrease in the C_H and C_{HO}.

It seems very unlikely from our knowledge of reactions in general that the rate of a reaction should be quantitatively proportional to the hydrogen ion concentration over a wide range and then suddenly become independent of it, to become later directly proportional to the hydroxyl ion concentration.

If we assume temporarily that the rate really is proportional to the hydrogen and hydroxyl ion concentration and that the discrepancy between the observed rate and the rate calculated on this basis is due to a change in the gelatin, we must assume that the gelatin is changed to a form which is very much more rapidly hydrolyzed. The value of the expression $\frac{\textit{observed velocity}}{C_H + 30\ C_{OH}}$ will evidently be a measure of this change. In Fig. 6 the logarithms of the values of this expression are plotted over the pH together with the titration

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curve of gelatin (taken from the titration curve for gelatin determined by Loeb), expressed as the per cent of uncombined gelatin present. It is apparent that the two curves are strikingly similar. Both are parallel to the X axis below pH 2 and then rise to a maximum, one at pH 5.0 and one at pH 6.0. Both then drop rapidly and again become parallel to the X axis beyond pH 11.0. This renders it probable that the rate of hydrolysis is some function of the amount of uncombined gelatin present. It will be noted from the table that the value of the "constant" at pH 5.0 (where the gelatin is completely uncombined) is about 200 times that of its value in the range where the gelatin is all present in the form of salt. If we assume then as a first approximation that the uncombined gelatin

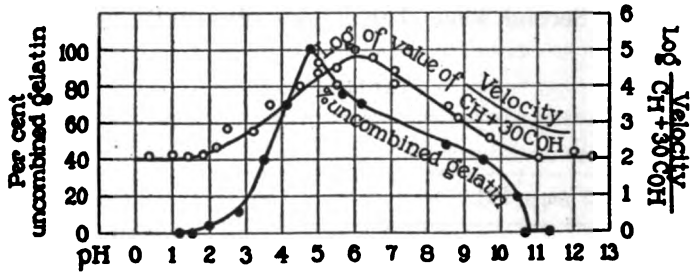

FIG. 6. Influence of the hydrogen ion concentration on percentage of uncombined gelatin and on the value of $K_1 = \frac{\text{elocity}}{C_H + 30C_{OH}}$.

hydrolyzes 200 times as rapidly as the combined and that the relative efficiency of the H and OH ions remains the same we get for the velocity of the reaction

$$\text{Velocity} = K_2 (C_H + 30\ C_{OH})\ (C_{combined} + 200\ C_{free}) \qquad (2)$$

where $C_{combined}$ and C_{free} refer to the concentration of free and combined gelatin as determined from the titration curve. The sum of these two quantities is taken as 1. The values for K_2 are given in the last column (Table VIII). It is evident that although they can hardly be considered as constant they are very much more so than the values for K_1. The rates calculated from (2) are plotted in Fig. 5. They may be considered satisfactory as a rough approximation.

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It must be remembered that a slight error in the pH determinations causes a very large variation in the constant and that a shift of the steeper portions of the titration curves of less than 0.2 pH will make a difference in the value of K_2 of several hundred per cent. It must also be noted that the values on the figure are plotted as the logarithms so that the discrepancies are larger than they appear.

It may be pointed out that a reaction very similar to the above was studied by Clibbins and Francis[23] in connection with the hydrolysis of nitrosotriacetoneamine. In this case also the reaction is directly proportional to the C_{OH} over a wide range and then becomes nearly independent of it. In still more alkaline solution the velocity becomes inversely proportional to the C_{OH}. Clibbens and Francis consider also that this is due to some change in the condition of the substance undergoing the reaction but were unable to account for it quantitatively. Many reactions have been studied in which the velocity of reaction of the ions was very different from that of the free substance. Richards and Stull[14] found that, in the reaction between oxalic acid and bromine, the divalent oxalate ion reacts very much more rapidly than any of the other forms present. Stieglitz[24] accounted for the mechanism of the hydrolysis of the imido-esters by the hypothesis that the ester-salt hydrolyzed either more or less rapidly than the free ester. Similar explanations have been proposed by Acree, Goldschmidt, and others.

It will be noted that the peculiar results obtained in the present work are all in the range of acidity where the enzymes are active and where the growth of microorganisms is possible. It seemed possible, therefore, that the increased rate in this range might be due to traces of enzymes or to the presence of bacteria or the action of the various preservatives. The experiments were therefore repeated at a temperature of 65°, without the addition of preservatives. Any traces of enzymes present would be inactivated very rapidly at this temperature and their effect would be noticed only for the first few hours at most. No such effect was noted. The

[23] Clibbens, D. A., and Francis, F., *J. Chem. Soc.*, Tr. 1912, ci, 2358. Francis, F., and Geake, F. H., *Soc., J. Chem.* Tr. 1913, ciii, 1722.

[24] Stieglitz, J., *Am. Chem. J.*, 1908, xxxix, 29.

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results of this series are given in Fig. 7. The pH measurements were made at 25° and calculated to 65° assuming the constant for water as 10^{-13} at this temperature and that the hydrogen ion concentration remains constant up to pH 8.0, and the hydroxyl ion concentration constant above pH 11.0. The curve is similar in every respect to that for 40°. There seems no possibility, therefore, that the anomalous rate of hydrolysis between pH 2.0 and 10.0 can be due to enzymes or microorganisms.

It will be noted that the rate of hydrolysis is 10 times more rapid at 65° than at 40° if solutions having the same hydrogen ion con-

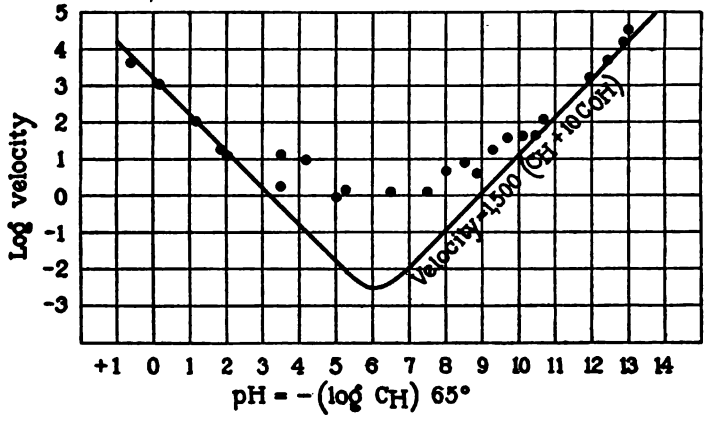

FIG. 7. Influence of the hydrogen ion concentration on the velocity of hydrolysis at 65°C.

centration at both temperatures are compared, but only 3 times more rapid if solutions having the same hydroxyl ion concentration are compared. This latter value seems remarkably low. A similar peculiarity was noticed by Michaelis and Rothstein[25] in studying the influence of the C_{OH} on the destruction of pepsin. These authors found that the destruction of pepsin was proportional to the third power of the C_{OH}, and that, if this was taken into account, the temperature had no effect on the velocity of the reaction.

Mechanism of the Reaction.—It was mentioned in the beginning of the paper that the hydrogen ion concentration decreases rapidly

[25] Michaelis, L., and Rothstein, W., *Biochem. Z.*, 1920, cv, 60.

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unless some means are provided such as buffer solutions for keeping it constant. The same is true for the hydroxyl ion concentration. According to the classical definition, therefore, the reaction is not catalytic since some of the catalyst combines with the products of the reaction and so would effect the final equilibrium. It seems probable that this is always true to some extent and that as was emphasized by Stieglitz, a "catalytic" reaction is merely the limiting case of an ordinary reaction in which the products of the reaction dissociate more or less completely liberating more or less of one of the original substances (cf. also Lewis[26] and Falk[27]). If the dissociation is complete no change could be detected in the concentration of one of the reacting substances and the reaction would be monomolecular in regard to the other. If there were no dissociation the reaction would, of course, be bimolecular. The present reaction is intermediate between the two. It is exactly analogous in this respect to the hydrolysis of gelatin by pepsin.

SUMMARY.

1. The hydrolysis of gelatin at a constant hydrogen ion concentration follows the course of a monomolecular reaction for about one-third of the reaction.

2. If the hydrogen ion concentration is not kept constant the amount of hydrolysis in certain ranges of acidity is proportional to the square root of the time (Schütz's rule).

3. The velocity of hydrolysis in strongly acid solution (pH less than 2.0) is directly proportional to the hydrogen ion concentration as determined by the hydrogen electrode *i.e.*, the "activity;" it is not proportional to the hydrogen ion concentration as determined by the conductivity ratio.

4. The addition of neutral salts increases the velocity of hydrolysis and the hydrogen ion concentration (as determined by the hydrogen electrode) to approximately the same extent.

[26] Lewis, W. C., McC., A system of physical chemistry, 2nd ed., London, New York, Bombay, Calcutta, Madras, 1918–19, i, 416.

[27] Falk, K. G., The chemistry of enzyme actions, American Chemical Society Monograph Series, New York, 1921, 33.

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5. The velocity in strongly alkaline solutions (pH greater than 10) is directly proportional to the hydroxyl ion concentration.

6. Between pH 2.0 and pH 10.0 the rate of hydrolysis is approximately constant and very much greater than would be calculated from the hydrogen and hydroxyl ion concentration. This may be roughly accounted for by the assumption that the uncombined gelatin hydrolyzes much more rapidly than the gelatin salt.

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ENERGY AND VISION.

By P. LECOMTE DU NOÜY.

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Although a large number of papers and books have been published on the problems of vision (1), a very limited amount of work is to be found on the minimum energy necessary to produce visual sensation. The classical work of Langley (2) for different wave lengths the papers of Grijns and Noyons (3), Zwaardemaker (4), and Kries (5), for white light are always quoted, but the figures given by different authors do not always agree, discrepancies of 100 per cent, sometimes of 1000 per cent, being frequent, with no explanation. For this reason, it was thought advisable to check all these figures, in order to ascertain whence came the discrepancies. Furthermore, as Langley's figures are given by himself with a certain degree of approximation, and were calculated for the light emitted by the sun, we thought it would be interesting to check them by another method, for another source of light, the Nernst lamp, for instance. These are the reasons for carrying on this series of measurements.

In order to give an idea of how difficult it is to find a figure corresponding to the *minimum visibile* for a certain wave length, we will give an example. Langley's figures are quoted as follows for the wave length 0.55μ, for which the human eye shows a maximum of sensitivity:

By Broca (6)..... 5.6×10^{-9} ergs
By Henri and des Bancels (7)..... 3.0×10^{-8} ergs

whereas Langley's *real* figure, as given in his paper, is 2.8×10^{-9} ergs. Furthermore, Henri and des Bancels state on another page that 10^{-10} is the order of magnitude of the minimum energy necessary to produce the sensation of vision in the green (0.55μ), and Langley (2)[1] states that it is 1.0×10^{-8} (for practically the same radiation, 0.53μ). In order to clear this matter up, we have to go over Langley's paper carefully. Langley states and gives a solution for two different problems: first, determination of the intensity of light necessary to read a table

[1] Langley, (2), p. 23.

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of logarithms or to discern any arbitrary character and second, determination of the *minimum visibile*; namely, the minimum of energy which can produce the sensation of light on the retina. The results of the first determinations are expressed in tables (2)[2] in function of the wave length and of the sensitiveness of the eye, in arbitrary figures related to the apparatus and inversely proportional to the energy. The results of the second determinations are expressed, on the contrary (2)[3] in the following way: reciprocals of calories = reciprocals of ergs (let us call this Table A), and these values stated in terms of horse-power (Table B). Now the meaning of these tables is quite ambiguous, and it is not surprising that authors have been mistaken in quoting them because, as they are given, they are only consistent provided the first table (A) is given in ergs per $\frac{1}{2}$ second. But as the figures in Table B, being expressed in horse-power, cannot be given in $\frac{1}{2}$ seconds (as the horse-power unit carries in itself its time unit and can only be used in connection with it, namely, 1 second), the figures of Table A must first be transformed into ergs per second, that is, multiplied by 2, to be identical with those of Table B. This is what has probably escaped the attention of Broca, and of Henri and des Bancels, and unfortunately, Broca took his figures from Table B, and Henri and des Bancels took theirs from Table A, so that all the figures of Broca are exactly double those of Henri and des Bancels. It is possible that these authors have not been mistaken, and that one of them (Broca) reduced the figures in Table A to ergs per second, whereas Henri and his coworker simply took them as they were. But it is most regrettable that none of them gave any indication as to the unit of time. Moreover, an important error is to be found in the figures of Henri and des Bancels due perhaps to misprint: for the wave length 0.55μ, they quote 3.0×10^{-8} ergs, (7)[4] instead of exactly 2.77×10^{-9}. If 3 may be taken as a roughly rounded figure for 2.77, however, the order of magnitude is different. In Broca's quotation, another error or misprint is also to be found: 3.6×10^{-3} ergs for 0.75μ, instead of 2.56×10^{-3}.

It may be of interest to compare the tables published by Broca, and Henri and des Bancels with the exact figures of Langley:

TABLE I.

	Langley.			Henriand des Bancels.	Broca.
	Reciprocal of				
				ergs	*ergs*
0.40μ	$1{,}500{,}000 \frac{\text{ergs}}{\frac{1}{2}\text{ sec.}}$	$= 6.7 \times 10^{-7} \frac{\text{ergs}}{\frac{1}{2}\text{ sec.}}$	$= 1.33 \times 10^{-6} \frac{\text{ergs}}{\text{sec.}}$	6.7×10^{-7}	1.37×10^{-6}
0.55μ	360,000,000 "	$= 2.77 \times 10^{-9}$ "	$= 5.55 \times 10^{-9}$ "	3.0×10^{-8}	5.6×10^{-9}
0.65μ	1,600,000 "	$= 6.29 \times 10^{-7}$ "	$= 1.26 \times 10^{-6}$ "	6.0×10^{-7}	1.26×10^{-6}
0.75μ	780 "	$= 1.23 \times 10^{-3}$ "	$= 2.56 \times 10^{-3}$ "	1.3×10^{-6}	3.6×10^{-3}

[2] Langley, (2), pp. 12, 13, 15.

[3] Langley, (2), p. 20.

[4] Henri and des Bancels, (7), p. 845.

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Langley gives another series of figures (2)[1] by which he intends to express "the proportionate results for seven points in the normal spectrum, whose wave lengths correspond approximately with those of the ordinary color divisions, where unity is the amount of energy (about $\frac{1}{1000}$ erg) required to make us see light in the crimson of the spectrum near A." According to this definition, this scale corresponds to the *minimum visibile*.

Wave lengths	0.40	0.47	0.53	0.58	0.60	0.65	0.75
Luminosity (visual effect)	1.600	62.000	100.000	28.000	14.000	1.200	1

Expressed in negative powers of 10, in order to facilitate comparison, we have (unity being 10^{-3} ergs, no indication being given concerning the time):

Wave lengths	0.40	0.53	0.65	0.75
Energy	6.2×10^{-7}	1×10^{-6}	8.3×10^{-7}	1×10^{-6}

The first figure (for 0.40μ) agrees well with that given by Langley (2)[2] in his other tables. The second one (0.53μ) does not agree at all, and the slight difference in wave length cannot be regarded as the cause of the discrepancy. The third one agrees within 25 per cent and the last one also, approximately. We see no explanation for this discrepancy, which cannot be due to a misprint.

Therefore, it was desirable to settle the question, since Langley's data are so misleading that good authors have made errors simply in quoting them. Quite recently, Joly (8) published a very interesting article on a quantum theory of vision, and although he does not share Henri's opinion on the subject, quotes one of his figures, 5×10^{-12} ergs for the threshold of sensitivity for white light. Now, we have tried in vain to find such a figure in two of the papers of Henri and des Bancels, as the indications of the source are missing. As far as we know, they did not make any measurements themselves, but simply quoted those of Grijns and Noyons. They quote the figures given by Grijns and Noyons, 4.4×10^{-11} ergs. Even if we admit that only 10 per cent of the energy is radiated under the form of light (9), we obtain 3.96×10^{-12}, and not 5×10^{-12}. It is regrettable that Professor Joly did not give the bibliographic reference.

Method.

An integration method was used. In other words, a curve representing the intensities of the dispersed beam after its passage through the prism was plotted in function of the wave lengths on coordinate paper. It is clear that the area delimited by this curve and certain

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limits, arbitrarily chosen, that is to say, the integral of the curve between these limits, will express the total energy radiated. As the source yields in the same time invisible and visible rays, and as the methods used for measuring the radiation give us figures corresponding to the total radiation, R (visible + invisible), a segment extending between the limits of the visible spectrum must also be integrated. This latter integration gives the quantity of energy spread in the visible part of the spectrum; let it be L. Then the ratio of these two areas will be the luminous efficiency of the source, and will be expressed by $\frac{L}{R} = E$. The percentage of the visible to the invisible is now known. Let us call I the intensities in function of the wave lengths, λ_1 the lower limit of integration, λ_2 the upper limit of integration for the visible, then:

$$E = \frac{L}{R} = \frac{\int_{\lambda_1}^{\lambda_2} I d\lambda}{\int_{o}^{\infty} I d\lambda}$$

The quantity of energy spread by the slit over the visible spectrum being thus known, a suitable screening of each monochromatic light decreases its intensity until the threshold of sensitivity is reached. Knowing exactly the amount of energy absorbed by the screens, the amount which is allowed to pass may be calculated easily: it is the minimum energy necessary to produce visual sensation.

Technique.

Limits of Integration.—Limits of Total Radiation.—Lower limit: For most light sources, the energy in the ultra-violet is so small that the lower limit, 0.4 μ, may be taken as zero without any appreciable error. Gage (10) takes it as the limit in his study of the electric arc, which is one of the richest sources in ultra-violet. The Nernst lamp, on the contrary, yields very little ultra-violet radiation, and it was assumed that this limit could safely be taken. Upper limit: The plotting of energy distribution curves showed that above 7μ in the infra-red, the amount of energy radiated by the Nernst

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lamp was very small, as compared to that radiated beyond. From 7 μ to 10 μ, it amounts to less than 1 per cent of the total. As the other errors involved by the method are of a greater order of magnitude, it was adopted as the upper limit, for the total radiation.

Limit between the Red and the Infra-Red.—(Upper limit of integration of the visible spectrum). Langley, although he does not specify it, seems to have chosen 0.75 μ as the upper limit. Many workers have chosen 0.8 μ (as the eye is sensitive to the radiations up to 0.8 μ). Some have preferred 0.76 μ, others 0.7 μ. The reason for the importance of this determination is that energy increases very much between 0.7 μ and 0.8 μ, whereas the impression on the eye is very slightly changed. In other words, the shifting of the limit from 0.8 μ to 0.7 μ will change considerably the amount of energy spent in the visible spectrum, whereas the effect on the eye will hardly be noticeable, since it only brings in very faint, deep red rays which, if absent, do not modify one's impression appreciably. On the other hand, if it is sought to determine the minimum of energy necessary to make the red rays between 0.7 μ and 0.8 μ impress the retina, one has to shift the limit as high as 0.8 μ. And in this case, all the values given for the energy of radiations below 0.7 μ will be altered (by more than 27 per cent). Therefore, in this paper, the two figures are given, so that one may compare the results.

The study of luminosity curves shows that, by removing the part of the spectrum extending beyond 0.7 μ, the total luminosity is only decreased by 0.4 per cent. As König and Brodhun (11) have shown that the human eye was just able to detect a change in luminosity when it amounted to 1.6 per cent, we feel that this limit is advisable.

Measurement of Total Radiation.

The first step was to measure the value in absolute units of the total radiation of the Nernst lamp, with which it was intended to experiment; for it was difficult, owing to the discrepancies found in the figures given by different authors, Lux (12), Hartman (13), Ingersoll (14), etc., to rely upon data found in literature.

The source was an ordinary Nernst lamp, (110 volts, 1.3 amperes). In order to prevent any fluctuations due to cooling by air currents, the glower was enclosed in a brass chamber, with just one rectangular

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slit (20 + 3 mm.), in front of the glower. The reflection from the heater and porcelain support was suppressed by fixing the glower by means of its platinum wires at the end of two leads. The inside of the chamber was blackened with soot. A voltmeter was placed across the terminals, and an ammeter in series, so as to know exactly the input in watts. Under normal conditions, it was found to be 87.5 volts $\times$ 1.05 amperes = 91.875 watts. These 91.875 watts are not all transmitted by radiation, but part of them are taken away by conduction and convection by the air. Lux and others give the ratio $\frac{\text{input}}{\text{radiation.}}$. But as these figures may correspond to different types of lamps, it was found safer to measure it directly. Besides, this would allow us, by a simple calculation, to check our radiation data against those published previously on the Nernst lamp.

It was first attempted to use a specially made mercury thermometer, with a known weight of mercury in a known weight of glass, blackened on the bulb of which the rays emerging from a 0.1 sq. cm. slit were concentrated by means of a fluorite lens of short focus. This process showed a lack of sensitivity and it was necessary to check it by means of an electric method. Although less difficult to handle than a bolometric device, the following apparatus required a great deal of care and time. A thermopile was made of copper and constantan wires, with ten elements, disposed linearly; the cold ends were simply bent out. On the top of the welded ends, carefully planed and ground, a thin piece of tin-foil exactly 1 mm. wide and 1 cm. long was applied and fixed with a very thin layer of shellac. Then the tin-foil was cut carefully between the welded ends, leaving a little square table of very nearly 1 sq. mm. on each thermocouple. These were blackened with soot, and the whole thermopile fixed in a thermostat. The rays were allowed to fall on the pile through an adjustable slit, and the distance between the source and the couples made equal to 1 meter. The method consisted in compensating the heat generated by the incoming radiation, by the current sent in a strip of constantan placed near the cold ends of the thermocouples, in a tiny calorimeter, 2 cc. in capacity, filled with oil, and well isolated. The following formula was used:

$$E = Ki^2 \frac{\text{cal. gr.}}{\text{0.1 sq. cm. sec.}}$$

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K being a constant (function of the resistance of the constantan strip) of the instrument, calculated and experimentally checked, equal to 0.21, we measured a current of 0.0028 amperes; this gives:

$$0.21 \times (0.0028)^2 \frac{\text{cal. gr.}}{\text{sec. } 0.1 \text{ sq. cm.}} = 0.00000165$$

as 0.2388 cal. gr. = 1 watt sec., it corresponds to 0.000694 watts by sq. cm.

Correction for Equatorial Radiation.—This corresponds to the homogeneous radiation of a punctual source of energy of 87 watts; that is, it would require 87 watts from a punctual source to radiate spherically in all directions an amount of energy of that magnitude. We have measured this amount equatorially, that is, normally to a line normal to the glower itself, and, of course, in the best conditions of radiation. But as the beam of light assumes a greater deviation from the equatorial plane, in the case of an incandescent rod, in other words, as the square centimeter exposed to the rays stands higher in latitude on the sphere, the amount of energy radiated is decreased, since the rays are no longer emitted perpendicularly by the rod. Around the two poles, there is even a region where there is no radiation at all. The result is that, whereas the source acts as radiating 87 watts equatorially, it radiates much less as soon as we reach higher latitudes, and becomes zero at the poles, and the mean value of the radiation is much less than 87 watts. It is known that by multiplying the energy radiated equatorially by $\frac{\pi}{4}$, the real value of the radiating energy from the source is known. In this case, $87 \times \frac{\pi}{4} = 68.3$ watts.

Hence, out of the 92 watts sent into the filament, only 68.3 are radiated, and 23.7 are lost by convection and conduction. The ratio $\frac{92}{68.3} = 1.35$ is in excellent agreement with the figure given by Lux: 1.34.

This figure may be checked in another way: the input in the filament being 91.8 watts, roughly 92 watts, it will radiate equatorially $92 \times \frac{4}{\pi} = 118$ watts,

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approximately. The quantity radiated actually as measured equatorially, being 87 watts, the ratio $\frac{118}{87} = 1.35$ gives the amount of energy lost.

Calculation of the Luminous Efficiency, and Corrections.—The next step was to determine the ratios

$$\frac{\int_{0.4}^{0.7} I d\lambda}{\int_{0.4}^{7} I d\lambda} \text{ and } \frac{\int_{0.4}^{0.8} I d\lambda}{\int_{0.4}^{7} I d\lambda} = \frac{L}{R}$$

This ratio has been calculated by many authors, Lux, Nichols and Coblentz (15), Ingersoll, Ångström (16), Stewart and Hoxie (17), etc. Their methods were different and their results do not always check perfectly, (some varying by more than 50 per cent, for example, those of Lux and Ångström.) Some of the workers used methods based upon the absorption of one part of the spectrum by water cells in which different substances were dissolved, (copper sulphate, iodine). It has been shown by Nichols and Coblentz that none of these methods based on absorption were reliable. Ingersoll studied the Nernst lamp and published figures of observed luminous efficiency, which vary greatly according to different lamps, and besides, correspond to burners whose consumption was not that of our lamp, (89 watts). Therefore, the energy distribution curve of our burner was plotted by means of a Hilger Infra-Red Spectrometer.[5]

Correction for the Non-Normal Spectrum.—The spectrum was corrected for the lack of homogeneity of the dispersed beam. Indeed, the refracted rays are contracted in certain parts of the spectrum, and expanded in others, so that, for instance, the same slit opening (*e.g.*, 0.25 mm.) covers 0.015 μ on the spectrum at a mean wave length 0.68 μ (from 0.6725 μ to 0.6875 μ), and as much as 0.266 μ, more than 17 times as much, at the mean wave length 2.66 μ (from 2.53 μ to 2.79 μ). This correction was introduced by the consideration of the geometry of the screw motion, (pitch of screw in relation to

[5] Instrument No. 281, Rock salt prism, Angle 59°. 57′. 30″.

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rotation of the prism table), and the use of the dispersion formula, given by Paschen (18):

$$n^2 = a^2 + \frac{M_1}{\lambda^2 - \lambda_1^2} + \frac{M_2}{\lambda^2 - \lambda_2^2} - K\lambda^2 - h\lambda^4$$

Then, the range of the spectrum embraced by a given slit was checked by moving a spectrum line across the slit, and reading the result on the drum. The right edge of the *D* lines (sodium), for instance, was brought in contact with the right edge of the slit (0.25 mm. opening), and the reading made. Then it was moved toward the left until the whole *D* line just disappeared, and another reading made. The result was 0.008 μ. This was done for the lines of copper (0.4955 μ, 0.5292 μ), mercury (0.5461 μ), sodium (0.5893 μ), and cadmium (0.6439 μ). For the infra-red, the data are published by Hilger (19).

It was decided to take the area covered at 2.66 μ by a slit 0.025 mm. wide as unit, (0.026 μ) and to fix the slit in such a way, for every wave length, as to cover the same range. A high sensitivity Leeds and Northrup galvanometer was used in connection with the thermopile, (galvanometer resistance = 12 ohms).

Corrections of the Absorptions Due to the Spectrometer.—But, before integrating the plotted curve, another very important correction had to be introduced regarding the absorption by the golden mirrors, because the energy distribution curve does not correspond to the total amount received by the collimator slit, and because the absorption is much greater for short than for long wave lengths. Fig. 1 shows how the absorption varies for different wave lengths.

If R is the coefficient of reflection, and 3 the number of mirrors, the amount of energy reflected is expressed by

$$I = I_0 R^3$$

It is easily seen that for 0.5 μ, for example, only $\frac{1}{9.6}$ of the incident light is transmitted, and much less still for 0.4 μ.

The absorption by the prism amounts to very little. Theoretically, from the formula, $J = J_0 K^c$, where c is the length of the path of light in the rock salt and K a constant equal to 1 between 0 and 9 μ, it is equal to zero. We found that, practically, for the visible,

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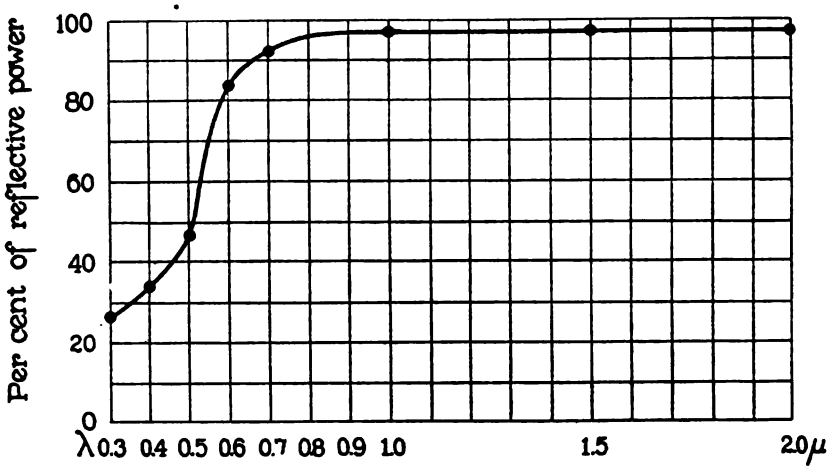

FIG. 1. Reflection of monochromatic light by gold.

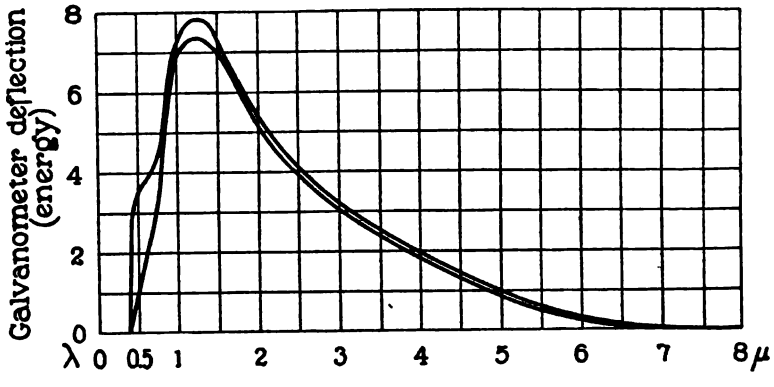

FIG. 2. Energy distribution curves for Nernst lamp, corrected and uncorrected for mirror absorption. Outside curve corrected; inside curve uncorrected.

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it really amounts to 1.5 per cent approximately, by concentrating the beam of light before and after passing through the prism.

Finally, the curves were integrated graphically. Fig. 2 shows the different aspects of the curves before and after corrections due to absorption. (These curves are not to scale, in order to emphasize the difference.) The results were:

$$R = \int_{0.4}^{7} = 110.3, \qquad L_{0.8} = \int_{0.4}^{0.8} = 4.8, \qquad L_{0.7} = \int_{0.4}^{0.7} = 3.6$$

The ratios are:

$$\text{Upper limit} = 0.8, \quad \frac{L}{R} = 0.0435$$

$$\text{Upper limit} = 0.7, \quad \frac{L}{R} = 0.0316$$

These results are in good agreement with those of Ingersoll, who, with the upper limit 0.76, found figures between 0.036 and 0.046, and in contradiction with those of Drude (20), who gives 12 per cent as luminous efficiency of Nernst lamps, (instead of 4.35 per cent). Lux gives 5.96 per cent, but takes no account of the fact that the screening method he used allowed most of the radiation up to 1.2 μ to pass. Coblentz and Nichols found 0.033 for the efficiency of acetylene flame.

Hence, as we have established that our source radiated 87 watts equatorially, the quantity radiated as luminous waves is equal to 4.35 per cent of 87 (limit 0.8 μ) = 3.8 watts, and 3.16 per cent of 87 (limit 0.7 μ) = 2.75 watts. Let us take the quantity corresponding to the limit 0.7 μ for example. These 2.75 watts are radiated at the distance of 103 cm. from the source, (distance of the thermopile) by square centimeter. The energy then becomes 0.0000206 watts. The area of the slit being 0.1 sq. cm., it only receives 0.00000206 watt seconds, or 20.6×10^{-7} watts = 20.6 ergs. This quantity of energy is spread over the range delimited by the upper and lower limits of integration (0.4 μ to 0.7 μ) and the energy distribution curve corrected for the absorption by mirrors, and by the prism, that is, over a surface of 83 sq. cm., (obtained by graphical integration of the curve, Fig. 3). This corresponds, in the scale chosen, to 0.248 ergs by unit of surface. Similarly, between the limits 0.4 μ

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and 0.8 μ, we find 0.343 ergs by unit of surface. In order to determine the efficient amount of energy which will affect the eye, it will easily be seen that this quantity will only have to be multiplied by the segment delimitated on the same chart, by the two ordinates corresponding to the range covered by the slit on the spectrum and the energy distribution curve uncorrected for mirror absorption.

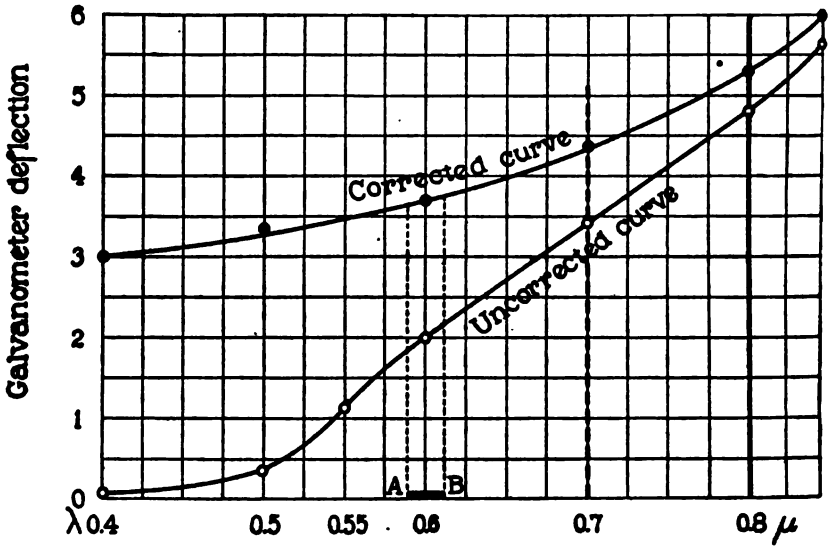

FIG. 3. Energy distribution curve, corrected and uncorrected, in a larger scale in the visible part of the spectrum. *A B* represents the area covered by a slit 0.25 mm. wide.

Test of the Eyes.

In order to reduce the intensity of light by a known quantity, a set of absorbing screens was prepared carefully. It was sought to look directly into the beam of light instead of using reflected light, in order to avoid the errors arising from the reflection of very faint radiations. By getting screens which could decrease by the same known amount, for example, 90 per cent, the intensity of the incident light, the simple formula

$$I = I_0 K^{-n}$$

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in which I = emergent light, I_0 = incident light, $K = \frac{I_0}{I}$ and n = the number of screens interposed, leads to this:

$$\text{Log } I_n = \text{Log } I - n \text{ Log } K$$

and as $K = 10$

$$\text{Log } I_n = \text{Log } I_0 - n$$

whence

$$I_n = I_0 \, 10^{-n}$$

The number of screens interposed will itself give the order of magnitude of the out-coming energy: 2 screens will mean that the energy is decreased by 100; 4 screens, by 10,000, etc.

It was found that especially prepared white paper fulfilled the requirements better than any other screen. Sheets of the same paper were chosen, (mean thickness 0.09 mm.), and placed exactly in front of the thermopile, then the throw of the galvanometer was observed; the paper was removed and another reading made at three different wave lengths. A great number were tested, and as we were unable to get ten sheets exactly similar, the thicker were placed on a plane surface and evenly rubbed down with very thin sand, then glossed again with a piece of round glass. They were frequently tested during this process, and finally the following results were considered as satisfactory. (The figures express the ratio $\frac{I}{I_0}$).

TABLE II.

Sheets.	0.55μ
No.	
1	0.100
2	0.103
3	0.103
4	0.095
5	0.0995
6	0.105
7	0.100
8	0.0965
Mean value.....	0.1005

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This particular paper was less transparent for the extreme red than for the green, a fact which had to be taken into account.

Measurements.—Eighteen persons were examined; two series of experiments were performed: one after 8 minutes in the dark, and one after 25 minutes. Only five persons were examined for all wave lengths. The others were merely tested for the radiation 0.55. The measurements were carried on in the following way: When nine sheets of paper were placed exactly against the slit, generally no light could be seen. Then, one after the other, the sheets were removed, according to the intensity of light, and usually, owing to the relatively large area (0.1 sq. cm.), there was no difficulty whatever in determining the order of magnitude of the *minimum visibile.* Namely, one sheet added gave a black impression, and this sheet removed left a visible, although very faintly colored, image of the slit. We sought, as Langley did, to determine the *minimum visibile,* defining this to be, not the smallest light whose existence it is possible to suspect, or even to be reasonably certain of, but a light which is observed to vanish and reappear when silently occulted and restored by an assistant without the observer's knowledge (Fig. 4).

On top of the last sheet of paper, another slit was placed, across the first one. Its jaws were cut in such a way that a square opening was left between them, (see Fig. 5); thus a square or rectangular figure was delimited by four moving lines. At first, the slit was adjusted so as to cut a little window of 1 sq. mm. on the luminous spot. If the window could not be seen, the jaws of the slit were moved micrometrically until the spot became visible. The maximum opening corresponded to a vertical motion of 5 mm.

It was found that most women generally require more time than men to reach the same degree of sensitivity. Most of the men tested became adapted in 5 minutes (viz., could see a light corresponding to an energy of the order of magnitude 10^{-9}) while it required 15 to 20 minutes for women to see the same thing. Moreover, some of them could only see the spot spasmodically appearing and disappearing, while men had a continuous impression. Only an increase in the intensity of about 50 to 100 per cent was able to give them the same visual impression. As will be seen, the differences vary between 0 and 25 per cent among men for a given wave length;

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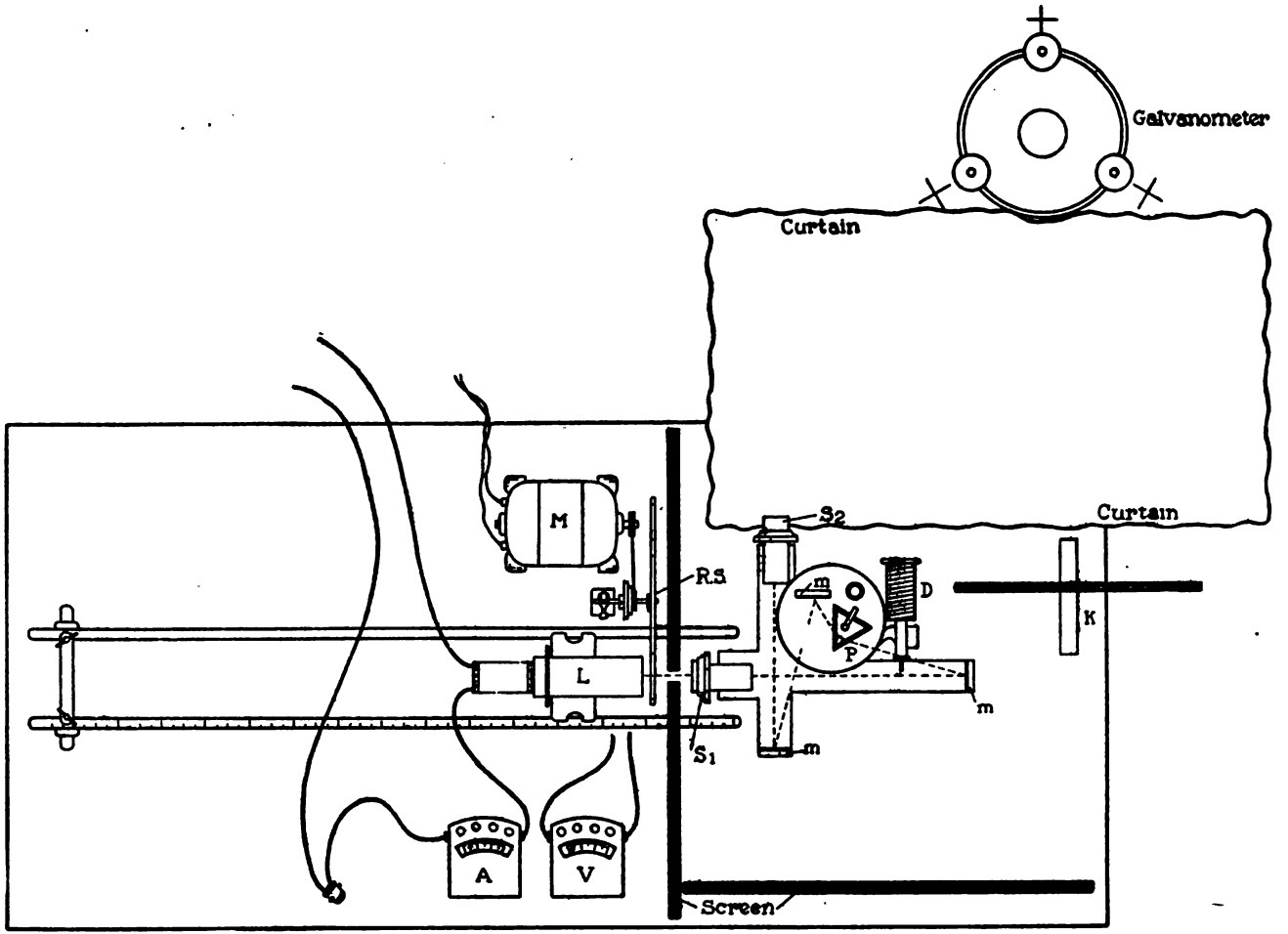

FIG. 4. Apparatus set for the test of the eyes. The observer was looking through slit S_2. *L*, Nernst lamp; *M*, motor; *R. S.* revolving shutter; *A*, ammeter; *V*, voltmeter; S_1, S_2, slits; *P*, rock salt prism; *D*, calibrated drum; *m*, *m*, mirrors; *K*, lamp and scale.

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among women, between 0 and 100 per cent. One man showed a marked difference.

The figures express the energy necessary for continuous impression but by making the same assumption as Langley concerning the

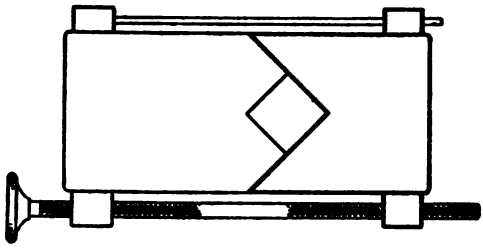

FIG. 5. Adjustable window.

minimum amount of time necessary to perceive distinctly a very faint light, (about $\frac{1}{2}$ second), these figures may be expressed in ergs per $\frac{1}{2}$ second, by dividing them by 2.

The size of the retinal image was approximately 0.01 sq. mm. ± 0.002.

TABLE III.

Men.

Observer.	Age.		0.4 to 0.8 μ	0.4 to 0.7 μ
	yrs.		$\frac{ergs}{sec.}$	$\frac{ergs}{sec.}$
A	29	Normal sight.	7.1×10^{-9}	4.55×10^{-9}
B	36	" "	8.2×10^{-9}	5.9×10^{-9}
C	45	Short sighted.	7.1×10^{-9}	4.55×10^{-9}
D	36	" "	8.3×10^{-9}	5.9×10^{-9}
E	28	Normal sight.	8.3×10^{-9}	5.95×10^{-9}
F	23	" "	8.1×10^{-9}	5.95×10^{-9}
G	19	" "	8.6×10^{-9}	6.1×10^{-9}
H	30	" "	7.4×10^{-9}	5.25×10^{-9}
I*	27	" "	1.0×10^{-8}	8.6×10^{-9}
Mean values.....			8.1×10^{-9}	5.85×10^{-9}

* Observer "I" was included in the mean, although he seemed to be quite out of the normal.

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First Series of Experiments.—The observers were protected by a curtain from all light, and waited until their eyes had become quite sensitive before making the experiments. 8 minutes in absolute darkness seemed to be sufficient for men. These first figures will show the difference between the rapidity of adaptation of men and women. Wave length 0.55 μ (Tables III and IV).

TABLE IV.

Women.

Observer.	Age.		0.4 to 0.8 μ
	yrs.		$\frac{ergs}{sec.}$
J	30	Normal sight.	8×10^{-9}
K	30	Short sighted.	2×10^{-8}
L	25	" "	9×10^{-9}
M	22	Normal sight.	1×10^{-8}
N	24	" "	2×10^{-7}
O	23	" "	3×10^{-8}
P	19	" "	2×10^{-8}
Q	42	" "	4×10^{-8}
R	40	" "	5×10^{-8}

Obviously, for women the differences are so great that a mean value would have no significance at all.

It was found that it took over 20 minutes for observers J, M, and O to reach the same sensitivity as men, viz., less than 7×10^{-9}. As Langley does not give any precision concerning the time of adaptation, we may compare his figures to the mean value found for men: Langley (0.55 μ), $5.55 \times 10^{-9} \frac{\text{ergs}}{\text{sec.}}$. We found slightly larger figures:

$$\int_{0.4}^{0.7} : 5.85 \times 10^{-9} \frac{\text{ergs}}{\text{sec.}}$$

$$\int_{0.4}^{0.8} : 8.1 \times 10^{-9} \frac{\text{ergs}}{\text{sec.}}$$

But after more than 20 minutes in the dark, the eye becomes more sensitive still, and we obtained the following figures (Table V). These figures are smaller than those given by Langley, but as he did not state the length of time which the eyes of his experimenters

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were kept in the dark, and as we have seen that the sensitivity is increased over 100 per cent by a stay of 25 minutes instead of 8 or 10, they cannot well be compared. Generally, at least, they are of the same order of magnitude for the wave length 0.55 μ. A stay of 1 hour in absolute darkness did not seem to increase the sensitivity beyond these figures.

It must be pointed out that the figures corresponding to the wave length 0.4 μ are doubtful, as the spectrometer which was used was not fit for the measurements in that part of the spectrum, owing to the gilded mirrors. They are only given as approximations. It must also be borne in mind that these quantities of energy do not

TABLE V.

25 Minutes in the Dark. $\int_{0.4}^{0.8}$

Observers.	0.4	0.5	0.55	0.65	0.68	0.72
(*Women*)						
J	2.5×10^{-6}	1.6×10^{-6}	3×10^{-9}	2×10^{-7}	2×10^{-6}	3×10^{-6}
O	5×10^{-6}	2.3×10^{-6}	4.5×10^{-9}	3.5×10^{-7}	5×10^{-6}	4×10^{-6}
(*Men*)						
B	5×10^{-7}	1.4×10^{-6}	2×10^{-9}	1.5×10^{-7}	1.9×10^{-6}	2×10^{-6}
D	5×10^{-7}	1.3×10^{-6}	2×10^{-9}	1.5×10^{-7}	2×10^{-6}	2×10^{-6}
F	8×10^{-7}	1.5×10^{-6}	3×10^{-9}	1.7×10^{-7}	2×10^{-6}	2.5×10^{-6}
Mean values...	3.85×10^{-7}	1.6×10^{-6}	3×10^{-9}	2.2×10^{-7}	2.7×10^{-6}	2.5×10^{-6}

correspond exactly to one pure radiation of wave length, 0.55 μ for instance, but to the beam comprised between 0.537 μ and 0.563 μ, the slit covering a range of 0.026 μ.

White Light.—The same technique was applied to the ***minimum visibile*** for white light (Nernst lamp), and gave 3.8×10^{-11} ergs, for continuous impression by total radiation. This figure agrees well with that of Grijns and Noyons for the Hefner lamp, 4.4×10^{-11}. It is better to compare figures related to total radiation, because these figures do not involve the more or less arbitrary choice of limits of integration and the knowledge of the ratio $\frac{L}{R}$ for the considered source.

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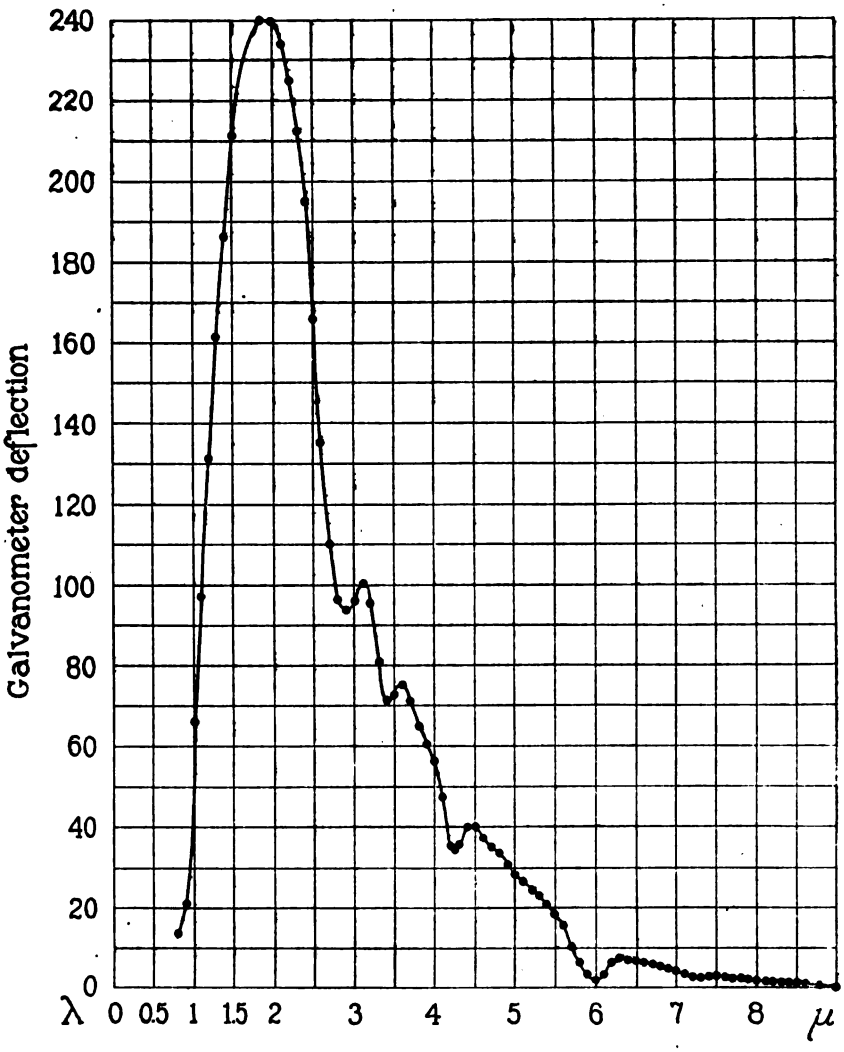

FIG. 6. Energy distribution curve of the Nernst lamp used (uncorrected), 91.8 watts: slits: 0.025 mm.

Criticisms of the Results.—As has been pointed out before by Langley, the errors involved in the determination of the threshold of sensitivity (*minimum visibile*) may be perhaps 100 per cent, or even more. For this reason, the absorption by the various eye

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media, for the total depth of the eye, which amounts only to about 1 per cent for 0.7 μ and less than 0.1 per cent below 0.65 μ, are entirely negligible. The eye, for such small amounts of energy as those corresponding to the *minimum visibile*, does not perceive a continuous increase in the brightness of the spot, when its intensity is increased progressively, but seems to react by steps. High authorities, such as Joly, and Henri, disagree entirely as to the explanation of vision on the basis of the quantum theory.

Accuracy of the Method.—In the method used, the following causes of error could be corrected:

Errors Due to the Spectrometer.

1. Selective reflection by the three gilded mirrors.
2. Selective absorption by the rock salt prism.
3. Contraction of the spectrum, (non-normal spectrum).

Errors Due to the Use of a Nernst Filament.

1. Uneven distribution of spherical energy.
2. Disturbing effect of volt- and ammeter in the circuit of the glower.
3. Radiant output of glower, (losses by conduction).

The following errors are also involved, and were not corrected:

Errors Due to the Integration Method.

1. Determination of limits of integration, (arbitrary).
2. Material errors due to the mechanical integration of surfaces.

We can probably admit that they do not amount to more than 10 per cent.

Errors Due to the Assumed Quantity of Energy Radiated.

1. Errors due to the fact that the image of the glower was not formed on the collimator slit.
2. Errors due to the emission of radiations from other parts of the instrument.
3. Errors due to the measurement of the total radiation by means of a compensating current.

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As our figures are in good accord with those of the best authors, within less than 10 per cent, we may assume that this is the upper limit of error. This gives a total of 20 per cent possible error, which is beyond the possibility of detection by the eye in the *minimum visibile*, as stated before. As some of the individual data differ by more than 100 per cent, the data can only be considered as reliable in the conditions of the experiments, within about 120 to 150 per cent. This is about the order of magnitude of the differences between the experimental data given by Langley.

Quantum Theory.—We can roughly express the *minimum visibile* in function of quanta of energy. For the mean radiation 0.55 μ, the period of the atom is 5.76×10^{14} per second. The minimum of energy perceived is approximately equal to 1.9×10^{-12} ergs per second, (taking 3.8×10^{-11} as the value of the minimum for total radiation, and roughly 5 per cent as belonging to the visible spectrum). Hence,

$$\frac{1.9 \times 10^{-12}}{5.75 \times 10^{14}} = 3.3 \times 10^{-27}$$

As Planck's universal constant $h = 6.5 \times 10^{-27}$, the figure found is satisfactory as far as the order of magnitude is concerned, but it would mean that only one-half quantum per second would be sufficient to cause the luminous sensation; as we have dealt with an area of $\frac{1}{100}$ of a square millimeter on the retina, it would indicate that the destruction of one molecule every 2 seconds on such an area would be sufficient to produce an impression of light.

CONCLUSION.

A method was devised for measuring the *minimum visibile* in different parts of the spectrum, as done by Langley in 1888.

The results are generally in good agreement with those given by this author, although not as close on both sides of the wave length 0.55 μ; this may be due partly to the use of a rock salt prism, to the fact that the minimum was determined by looking at a beam of diffused transmitted, instead of diffused reflected light, and also to the fact that Langley experimented with the sun, through the earth's atmosphere, and had to take into account the thickness of the atmos-

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phere interposed and the brightness of the sky. Although his experiments were made with great care, the differences from one day to another are important. However, when he expresses the energy in absolute units, he always refers to the same mean amount of energy radiated by the sun on 1 sq. cm. This amount is certainly not constant, if one judges from the differences observed in two measurements of sensitivity of the eye of the same individual at different dates. On the contrary, for a given wave length, our measurements always agreed closely, as our source of radiation was very nearly constant, owing to the absence of a varying amount of water vapor interposed. This may in some way account for the discrepancies observed.

I wish to express my thanks to Dr. Harry Clark of The Rockefeller Institute for the valuable help he was kind enough to give me in solving certain difficulties which were encountered during this work.

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17. Stewart and Hoxie, cited by Nichols and Coblentz, *Phys. Rev.*, 1903, xvii, 267.
18. Paschen, F., *Ann. Physik.*, 1908, xxvi, 130.
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THE RATE OF GROWTH OF THE DOMESTIC FOWL.

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(Received for publication, February 24, 1921.)

If the weight of a mammal, for example the mouse, is plotted against its age, the resulting curve will be seen to show three fairly distinct waves, oscillations, or growth cycles. These waves or cycles are still more strikingly shown by plotting the velocity of growth against age as is seen in Fig. 1.

Robertson[1,2] found that the growth of each of these cycles can be represented by the equation of an autocatalytic monomolecular reaction. This fact, and the theories developed from the fundamental studies on cell growth by Sachs, Morgan, Driesch, Boveri, and Loeb,[3] which were reviewed in a previous paper[4] on the growth of the dairy cow, led Robertson[1,2] and Ostwald[5] to assume that the limiting factor of growth of each cycle is an autocatalytic monomolecular reaction. Assuming this tricyclic and autocatalytic theory of growth to be true for mammals, it becomes of interest to find out whether the same does not also hold for birds. The object of this paper is to examine data on the growth of the domestic fowl in the light of this theory.

Fig. 2, deduced from Table I, shows the rate of growth of the Rhode Island Red breed of fowl[6] from the time of hatching up to the age of

[1] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363.

[2] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.* 1907–08, xxv, 581; *Am. J. Physiol.*, 1915, xxxvii, 1, 74; Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920.

[3] Loeb, J., The dynamics of living matter, New York, 1906, 58–66; *Biochem. Z.*, 1907, ii, 34; *Biol. Centr.*, 1910, xxx, 347.

[4] Brody, S., and Ragsdale, A. C., *J. Gen. Physiol.*, 1920–21, iii, 623.

[5] Ostwald, W., Vorträge and Aufsätze über Entwicklungsmechanik der Organismen, Leipsic, 1908, v.

[6] Card, L. E., and Kirkpatrick, W. F., *Storrs Agric. Exp. Station, Bull. 96*, 1918.

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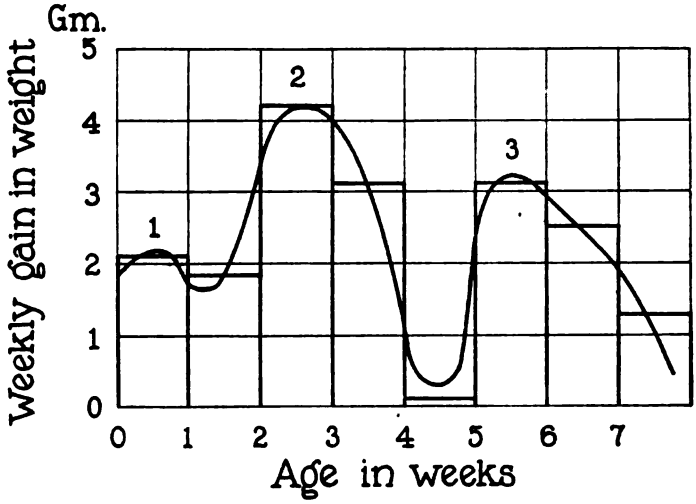

FIG. 1. The rate of growth of the white mouse plotted from data by Robertson.[1] Ordinates represent the weight (x) in grams gained per week; abscissæ represent the age (t) in weeks of the animals.

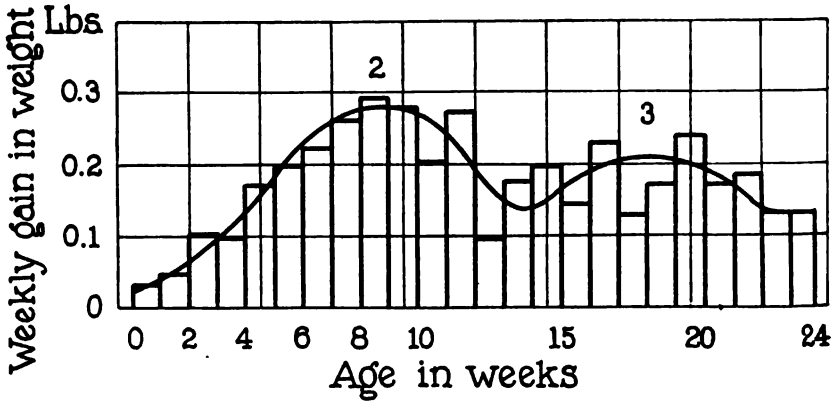

FIG. 2. The rate of growth of the Rhode Island Red fowl plotted from data by Card and Kirkpatrick.[6] Ordinates represent the weight (x) in pounds gained per week; abscissæ represent the age (t) in weeks of the birds from the time of hatching.

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laying. This figure clearly shows the rate of growth to increase from the time of hatching up to about 8.5 weeks of age when the rate of

TABLE I.

Growth of the Rhode Island Red birds.			Growth of chick embryo.			
After Card and Kirkpatrick.[6]			After Lamson and Edmond.[9]		After Hasselbalch.[10]	
Age.	Weight.	Number of birds.	Age.	Weight.†	Age.	Weight.
wks.	*lbs.*		*days*	*gms.*	*days*	*gms.*
0 (hatched)	0.082	865			3	0.004
1	0.115	858	4	0.12	4	0.054
2	0.162	798	5	0.28	5	0.155
3	0.264	750	6	0.49	6	0.373
4	0.364	714	7	0.86	7	0.615
5	0.538	696	8	1.35	8	1.200
6	0.737	682	9	1.98	9	2.040
7	0.962	677	10	2.85	10	2.890
8	1.228	613	11	3.95	11	4.366
9	1.525	611	12	5.89	12	5.674
10	1.805	608	13	8.21	13	7.543
11	2.014	601	14	9.84	14	10.005
12	2.290	600	15	12.63	15	12.285
13	2.388	344*	16	16.05	16	15.210
14	2.566	343	17	20.05	17	17.500
15	2.765	343	18	21.36	18	21.545
16	2.907	288*	19	26.87		
17	3.136	288	20	35.50		
18	3.265	288				
19	3.436	253				
20	3.677	252				
21	3.851	251				
22	4.035	250				
23	4.165	250				
24	4.296	250				

* The males were removed at the end of the twelfth and fifteenth weeks. The other reductions in number are due to removal of chicks by crows and necessity of removing chicks due to limitations of range conditions.

† Average of ten embryos.

growth is at a maximum; from this maximum at 8.5 weeks the rate of growth decreases to a minimum at about 14 weeks. This is followed by another cycle with a maximum at about 18 weeks of age ending

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somewhere between 24 and 28 weeks[6] of age when growth of non-fatty tissues probably ceases. That each of these postembryonic cycles follows the equation of an autocatalytic monomolecular reaction can be seen from their shape. This can be easily verified by taking t_1, the age of maximum growth at 8.5 weeks, and following the technique of computation described in the preceding paper on the growth of the dairy cow, we obtain the equation

$$\text{Log } \frac{x}{2.76 - x} = 0.182\ (t - 8.5)$$

which gives calculated values closely agreeing with the observed weights.

Age (t).	Observed weights.	Calculated weights (x).
wks.	*lbs.*	*lbs.*
0	0.082	0.078
1	0.115	0.114
2	0.162	0.168
3	0.264	0.251
4	0.364	0.363
5	0.538	0.516
6	0.737	0.717
7	0.962	0.961
8	1.228	1.233
9	1.525	1.521
10	1.805	1.800
11	2.014	2.040
12	2.290	2.240
13	2.388	2.390
14	2.566	2.505

Essentially similar results were obtained with other breeds of fowl such as the White Leghorn,[6] single comb White Leghorn,[7] and White Plymouth Rocks,[8] the several breeds differing only with respect to the several constants. From Fig. 2 it is clear that the equation will also hold somewhat less accurately for the second cycle with a maximum at 18 weeks.

[7] Lamon, H. M., and Lee, A. R., *U. S. Dept. Agric., Bull. 561*, 1917, 37.

[8] Phillips, A. G., *Purdue Univ. Agric. Exp. Station, Bull. 1906*, 1916; *Purdue Univ. Agric. Exp. Station, Bull. 214*, 1918.

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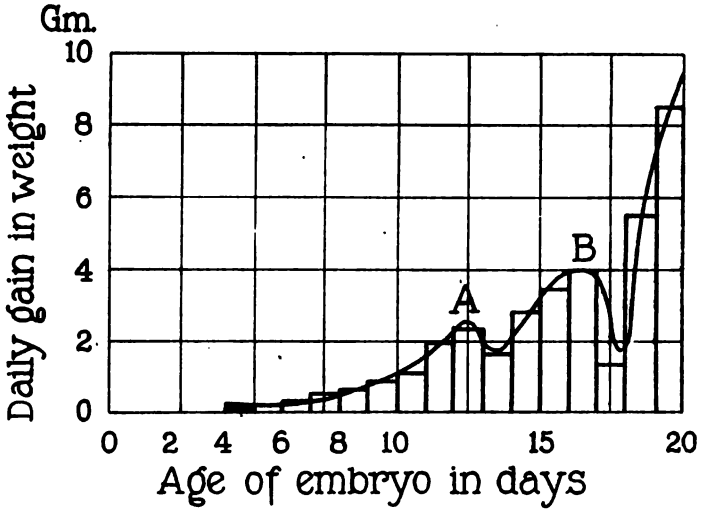

FIG. 3. The rate of growth of the chick within the egg plotted from data by Lamson and Edmond.[9] Ordinates represent the weight (x) in grams gained per day; abscissæ represent the age (t) of the embryo from the beginning of incubation.

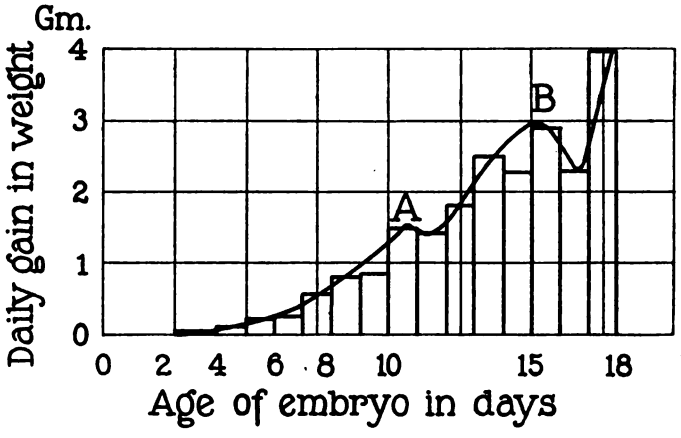

FIG. 4. Same as Fig. 3 plotted after data by Hasselbalch.[10]

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The mammal was seen to go through at least three cycles during its growth period; if the tricyclic theory of growth is also true for the bird, then the fowl must go through a complete cycle during its embryonic period of growth. Figs. 3 and 4, curves of the embryonic period of growth, show this to be the fact;[9,10] indeed it seems as if the curve of the embryonic period of growth shows two cycles with maxima at A and B.

SUMMARY.

This paper points out the fact that the growth period of the domestic fowl is analogous to that of the mammal, being composed of three, or perhaps four, cycles; two of these cycles are postembryonic with maxima at about 8 and 18 weeks varying somewhat with the breed and two or at least one, are embryonic with maxima at 11 to 12 and 15 to 16 days of age. Hatching occurs during the first part of the second or third cycle resembling in this respect the guinea pig[11] rather than the mouse.[1] The velocity curves of each of these cycles are similar to and can be represented by the equation of an autocatalytic monomolecular reaction.

The author is indebted to Mr. H. L. Kempster, Department of Poultry Husbandry of this Station for supplying him with some references in this paper.

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THE THERMOLABILITY OF COMPLEMENT, IN RELATION TO THE HYDROGEN ION CONCENTRATION.

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(Received for publication, May 12, 1921.)

That property of fresh blood serum by which it is able to take part in, and complete various immunological reactions is known as complement. The thermolability of this function is well known. In respect to the separated fractions, that contained in the euglobulin fraction of the serum and known as mid-piece, and that contained in the pseudoglobulin and albumin fraction and referred to as end-piece, Ferrata[1] found only the end-piece thermolabile. Subsequent observers have uniformly found both fractions thermolabile. A number of investigators[2, 3, 4] have noted that the mid-piece is more resistant when heated in whole serum than when isolated. Leschly[5] found moreover that the mid-piece, apparently inactivated by brief heating to 56°C. was able to complete hemolysis when added separately to the sensitized cells, the addition of end-piece being made after an interval, so that under these conditions there is not a permanent destruction, but the development of the modification described by Brand.[6]

With regard to the effect of the chemical reaction it has been known since the observations of Ehrlich and Morgenroth[7] and Ehrlich and Sachs[8] that the addition of considerable amounts of acid or alkali

*Van Cott Fellow in Pathology.

[1] Ferrata, A., *Berl. Klin. Woch.*, 1907, xliv, 366.

[2] Freidmann, U., *Z. Hyg. und Infektionskrankh.*, 1910, lxvii, 279.

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[4] Mutermilch *Compt. Rend Soc. de Biol.*, 1911, lxx, 577.

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[6] Brand, O., *Berl. Klin. Woch.*, 1907, xliv, 1075.

[7] Ehrlich, R. and Morgenroth, J., *Berl. Klin. Woch.*, 1899, xxxvi, 481.

[8] Ehrlich, R., and Sachs, H., *Berl. Klin. Wock.*, 1902, xxxix, 297.

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permanently inactivate complement. The destructive or inhibitory effect of slight degrees of acidity or alkalinity has been noted by a number of later workers. Liefmann and Cohn,[9] and Guggenheimer[10] found that the isolated mid-piece is more affected by the chemical reaction than is the end-piece; both fractions are more affected by acid than by alkali. Michaelis and Skwirsky[11] and Leschly[6] have worked with definite H ion concentrations, but their results do not relate to the present problem. Brooks[12] has recently defined the limits of pH value of the acidity beyond which complement is permanently inactivated.

The salt concentration of the solution has been found to affect complement in a way which appears to be quite different from that in which the two fractions of complement are separated by dilution with distilled water and dialysis, as in the original method of Ferrata.[1] Sachs and Teruuchi[13] found that when fresh serum is diluted 1 to 10 with distilled water a permanent inactivation occurs, and takes place more rapidly at 37°C. than at lower temperatures.

In the present work the degree of complementary activity retained by fresh guinea pig serum after being heated to various temperatures in solution in distilled water or in saline solution, at definite H ion concentrations, has been determined by a modification of the method of Brooks.[14] This consisted in measuring in a colorimeter to provide a standard for comparison the percentage of hemolysis given by the following amounts: 1, 0.8, 0.6, 0.4, 0.3, 0.2, 0.1, and 0.05 cc. of a dilution of each specimen of serum in saline solution. This dilution was first made 1 to 20, and then slightly increased to correspond exactly to the final dilution of the samples which were heated under varying conditions of temperature and reaction. The degrees of hemolysis thus observed were plotted as ordinates against the amounts of complement dilution as abscissæ. From the curve so obtained the relative efficiency or activity of each

[9] Leifmann H., and Cohn, M., *Z. Immunitätsforsch. Orig.*, 1910, vi, 562.
[10] Guggenheimer, H., *Z. Immunitätsforsch. Orig.*, 1911, xi, 393.
[11] Michaelis, L. and Skwirsky, P., *Z. Immunitätsforsch. Orig.*, 1910, vi, 357, 629
[12] Brooks S. C. *J. Gen. Physiol.*, 1919–20, 185.
[13] Sachs, H., and Teruuchi, Y., *Berl. Klin. Woch.*, 1907 xliv, 467.
[14] Brooks, S. C., *J Med. Res.*, 1919–20, xli, 399.

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heated sample can be obtained by reading the abscissa at which the percentage of hemolysis given by 1 cc. of the diluted and heated specimen intersects the base-line curve. The various corrections used by Brooks were not made. By the use of this principle as Brooks has shown, one compares amounts which produce like results, and can thereby obtain the actual ratio between the unheated control and the heated sample with respect to the complementary function under consideration. For each complete experiment there was used one stock emulsion of 5 per cent sheep cells sensitized with two units of sensitizer. The total volume in each tube of the titration and of the test was 5 cc. before the addition of acid or alkali.

The H ion concentration was determined by diluting three 2.5 cc. portions of each specimen of serum to 5 cc. with distilled water or saline, according to the medium chosen, adding methyl red and bromthymol blue separately to two of the tubes and determining by comparison with a standard series the H ion concentration reached after the addition of successive drops of N/40 HCl in equal number to each tube. The third tube served as a color screen after each addition of acid.[15] For each specimen of the isolated fractions of complement a similar titration was carried out, using for the end-piece N/40 and for the mid-piece N/160 HCl and NaOH.

The partition of the complement into the fractions was carried out by bringing the fresh serum diluted 1 to 20 with cold distilled water to a pH between 6.2 and 6.4, using for this purpose the necessary amount of HCl as calculated from the titration described above. This narrow range of reaction is that which was found optimal for the precipitation of the euglobulin under these conditions. A titration was found necessary for each specimen of serum because of differences in the amount of acid required to bring the pH to the desired reaction, and the blood of a single animal for the same reason was used for each experiment. The different methods which have been used for the partition of complement obviously depend upon the insolubility of the euglobulin within a narrow range of reaction

[15] Electrometric control of the colorimetric determinations indicate that the limit of error in the latter is pH 0.2. Between pH 5.8 and 6.5 the error is consistent in sign and lies between +0.15 and +0.10, the electrometric pH is more acid. No corrections have been made on the curves given here.

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in a salt-poor medium. After bringing the serum dilution to the desired reaction it was left standing 1 hour on ice and the flocculent precipitate brought down by centifugation. The supernatant fluid was decanted, the inner walls of the tube wiped dry with a clean cloth and the sediment representing the mid-piece used at once or after a single washing with distilled water of pH 6.0. The supernatant fluid from the first sedimentation was used as the end-piece dilution. The final preparation of mid-piece was made by carefully emulsifying the sediment in distilled water, or by subsequently bringing it into solution by the addition of NaOH to pH 7.4.

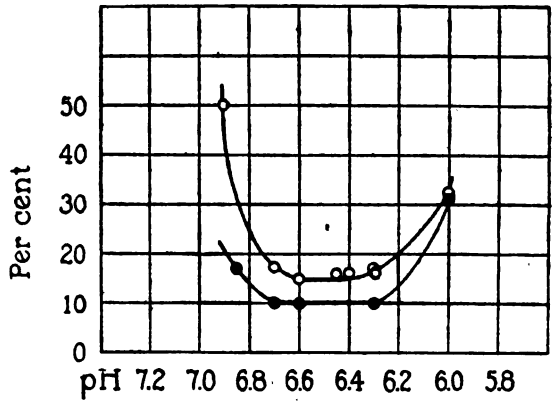

FIG. 1. The complementary activity of the supernatant fluid alone, after precipitation of the euglobulin at different reactions. One experiment is represented by the lower curve and two by the upper curve.

Many workers have had difficulty in separating completely the fractions of complement. The extent to which the completeness of separation depends upon the hydrogen ion concentration at which the separation takes place, is shown by the curve, Fig. 1. This records the complementary activity of the supernatant fluid alone separated at different reactions from the euglobulin sediment.

After adding the amounts of acid or alkali necessary to produce the desired reaction, to a series of tubes containing the different complement dilutions, the tubes were heated in all cases for 10 minutes in a water bath, then cooled by placing in ice water. The fluids in the tubes were all brought to the same reaction (pH 7.3 in the case of whole

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serum, pH 6.3 in the case of the fractions) the volumes equalized, and where distilled water had been used as the diluent, isotonicity was restored by the addition of one-tenth volume of 8.5 per cent NaCl solution. The original dilution was 1 to 20 in all cases, and the fractions were used in the same corresponding dilution. Between manipulations the tubes were placed in ice water and protected from sunlight.

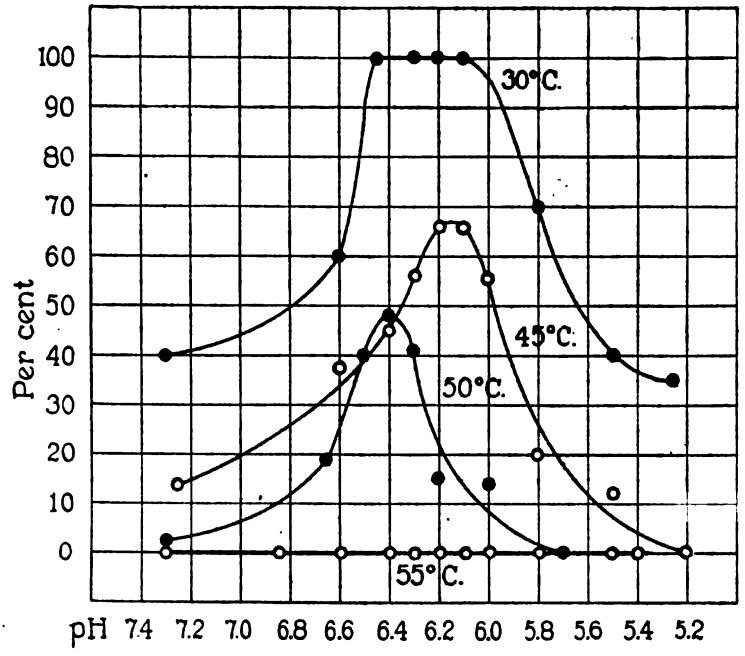

FIG. 2. The complementary activity retained by whole serum diluted in distilled water and heated at different temperatures, at varying reactions.

The complementary activity in percentages of that of the unheated control retained by whole serum diluted 1 to 20 in distilled water and heated at varying reactions at different temperatures, is shown in Fig. 2. The experiments recorded gave the highest values observed in many similar experiments. The value at any given pH relative to the unheated control varies with different specimens of serum, so that each curve is given to represent a single experiment. Although the amount of destruction increases with the temperature it is possible on account of this individual variation in thermolability,

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which has been noted by all observers, to make accurate comparison only of the relative degrees of inactivation at the different reactions. In all experiments it was found that the destruction of complement is least at a point between pH 6.1 and 6.4. Within this narrow range from 20 per cent to 50 per cent of the original, activity may be retained when the complement is heated to 50°C. for 10 minutes. On either side of the optimal reaction the drop in activity is rapid. The curves run approximately parallel for all temperatures of heating. At 53°C. a very small degree of hemolytic activity is retained at the optimal reaction; when heated to 55°C. for 10 minutes all complementary power is lost, when referred to the small amount of sensitizer used here, at all reactions.

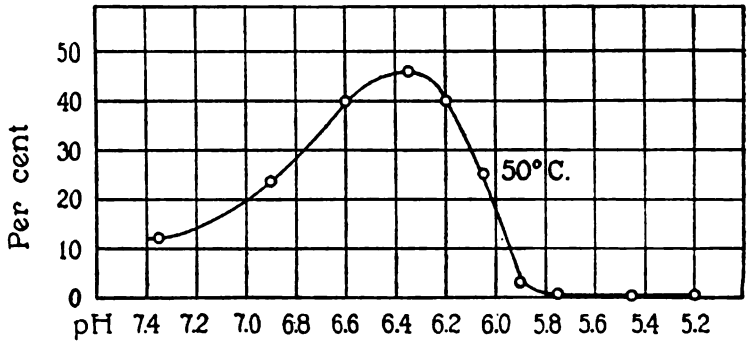

FIG. 3. The complementary activity of the euglobulin fraction in the presence of added end-piece, after heating whole serum to 50°C. in distilled water at the pH values given, and then separating the euglobulin.

If after heating the serum dilution the euglobulin is separated by bringing the reaction in all the tubes to pH 6.3 and removing the sediment by centrifugation, the complementary power of this euglobulin in the presence of the titer amount of end-piece follows the curve given in Fig. 3. It is evident then that the preservation of the complementary power at pH 6.1 to 6.4 depends upon preservation of the mid-piece function at these reactions.

A second maximum in the preservation of the complement was found in numerous experiments at pH 6.9, as shown in the lower curve in Fig. 4, from a typical experiment. The importance of this particular reaction was not appreciated in the early experiments

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in which it was not included. It depends apparently upon the greater preservation of the end-piece function at this reaction. This will be referred to later. If the heated complement be added, without separation into its fractions, to sensitized cells together with unheated mid- and end-piece in separate series, the upper curves of Fig. 4 result. The two maxima observed at pH 6.9 and at pH 6.1 to 6.4 appear to be an expression of the quantitative relations between the two fractions, according to which the degree of hemolysis is in-

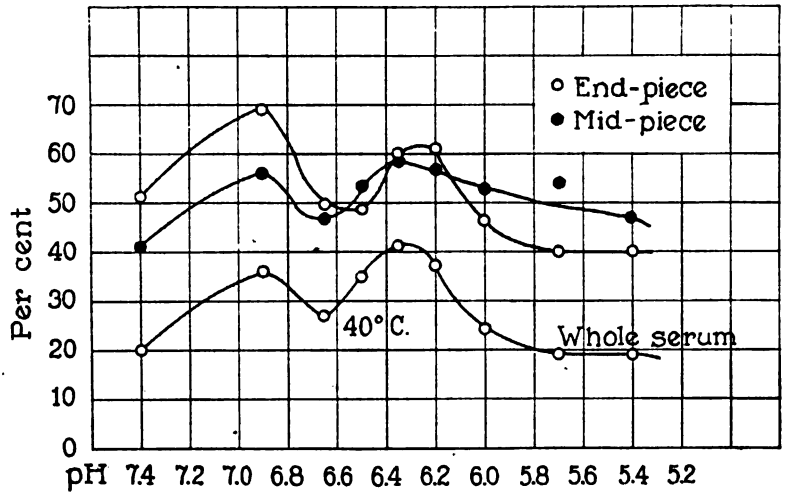

FIG. 4. The complementary activity of whole serum heated to 40°C. in distilled water dilution, by itself and in the presence of added mid- and end-piece. The curve marked end-piece is that given after the addition of the titer amount of mid-piece to each tube; that marked mid-piece is that given after the addition of the titer amount of end-piece to each tube.

creased if the amount of either mid- or end-piece is increased up to the titer value of each.[5]

The activity retained by the isolated end-piece fraction, freed from mid-piece by the precipitation of the euglobulin and heated in dilution in distilled water is shown in Fig. 5. In the experiments recorded here with the isolated fractions the original activity of the complement was completely restored on reuniting the unheated fractions, and the curves that show the effect of heating are essentially the same whether the activity is estimated from the base-line

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curve given by the unsplit and unheated control or from that given by varying amounts of one fraction and constant amounts of the other fraction, in each tube of the control titration. It is evident that there is a broad optimal zone for the preservation of the end-piece function, with a reaction of about pH 6.9 as its central and highest point. At this reaction the end-piece is relatively thermostable as compared with whole complement.

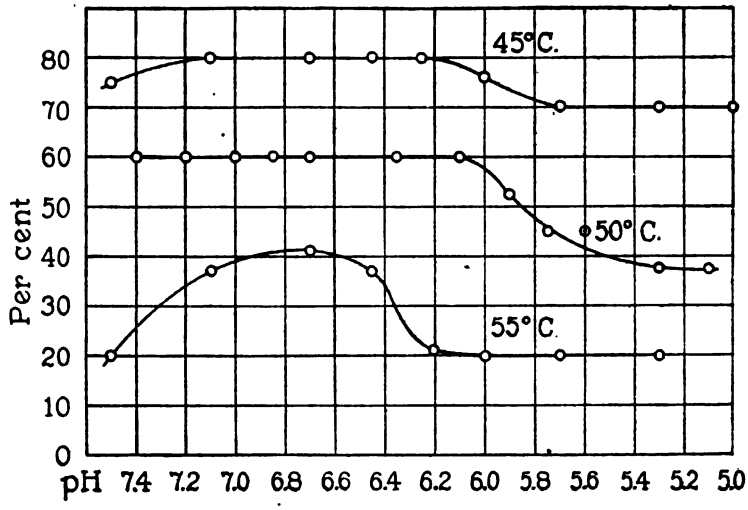

FIG. 5. The complementary activity in the presence of unheated mid-piece of the end-piece fraction heated separately in distilled water dilution at different reactions.

A similar relative independence of the H ion concentration as compared with whole complement is shown by the isolated mid-piece fraction, heated in distilled water. Two experiments at 50°C. are shown in Fig. 6. There is an optimal zone about pH 6.2 with a gradual fall on the alkaline side and a more rapid fall on the acid side of this point.

In the experiments with the isolated fractions the mid-piece whether heated or unheated was added first to the sensitized cells in order to avoid the peculiar modification described by Brand.[6] The curves represent therefore the degree of complete or irreversible inactivation of the fractions.

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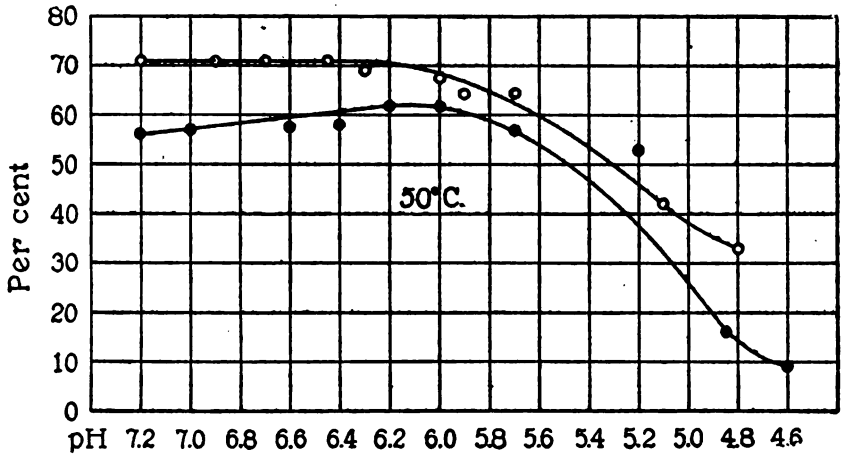

FIG. 6. The complementary activity in the presence of unheated end-piece of the euglobulin (mid-piece) fraction heated separately in distilled water at different reactions.

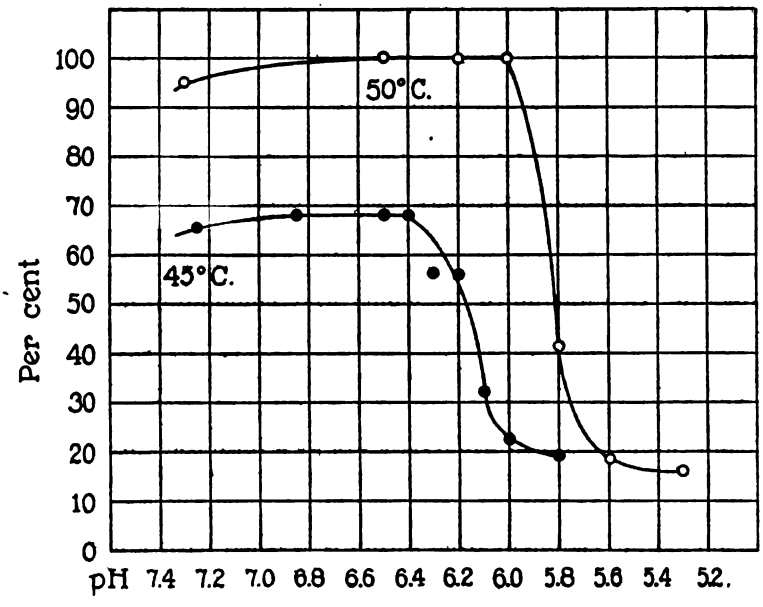

FIG. 7. The complementary activity of whole serum heated at different reactions in isotonic saline solution. The individual variation in thermolability is shown by the different maxima reached in the two experiments recorded here, the lower values being given in this case by the serum which was heated to the lower temperature.

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The mid-piece function is thus better preserved when isolated than when heated in the presence of the other constituents of serum at all the reactions except those in the narrow zone pH 6.1 to 6.4, within which it is almost equally thermostable in both cases.

If fresh guinea pig serum is heated in dilution in saline solution instead of in distilled water it is evident from Fig. 7 that the complementary function is better preserved and is less affected by change in reaction than when heated in distilled water, at reactions on the alkaline side of pH 6.1 to 6.4. On the acid side of this point the loss in activity with increasing acidity is as great in the presence as in the absence of salt. The determination of the H ion concentration is affected with a greater error in the dilution of the serum in saline than in distilled water, when colorimetric methods are used. Nevertheless the turning-point observed is essentially the same as in the experiments in distilled water dilution, and corresponds with the point on the curve given by Brooks[12] at which the destruction of complement begins in saline solution when the temperature is kept at 10°C.

DISCUSSION.

The inactivation which complement undergoes when heated in distilled water dilution is closely related to the properties of the euglobulin fraction since this destruction is least at the reaction at which the euglobulin is least soluble. The chemical reaction involved in thermolability in which the euglobulin is necessarily one of the reacting substances is therefore determined by the chemical state in which the euglobulin exists.

It has been found in the case of a number of proteins that solubility and other physical properties are at a minimum at the H ion concentration characteristic of the isoelectric point. At this point the ionization of the protein either as an acid or as a base is at a minimum. If the euglobulin sediment from serum is washed with a large volume of distilled water and brought into solution again by the addition of NaOH to pH 7.4 it becomes least soluble on the addition of HCl between pH 5.1 and 5.7 (depending probably on its purity) and is isoelectric about pH 5.0. The latter determination was made electrometrically; the value corresponds closely with that given by Rona

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and Michaelis[16] for serum euglobulin (about pH 5.2). When examined in whole serum however the euglobulin precipitates best at pH 6.3 as noted above and shows no movement in the electric field between pH 6.2 and 6.4. Under these conditions it exists therefore probably not as pure euglobulin but as a compound with some other substance of the serum.

Different compounds of a protein may differ in the position of the isoelectric point. When the reaction of red blood cell suspensions in saccharose solution is adjusted with NaOH and HCl the isoelectric point as determined by cataphoresis and the optimum for agglutination lie near pH 4.7;[17] in the presence of sodium acetate and sodium phthalate this critical point is found near pH 3.8.[18] The compound with a weak acid thus differs from the compound with a strong acid, as pointed out by Professor J. L. R. Morgan;[19] it is possible then that the value pH 6.1 to 6.4 represents the critical point in the ionization of a compound of the euglobulin with a weak base, in which state it exists in the serum or when dissolved or suspended in distilled water without washing. We may conclude then that it is chiefly or entirely the ionized fraction of the euglobulin which takes part in the reaction involved in thermolability.

The existence of a point of inflection in the thermostability of the end-piece at pH 6.9 suggests a similar interpretation in the case of this fraction. No other data are available however by which to identify the substance or compound concerned.

The difference in behavior of the fractions of complement when heated separately and when heated together suggests that during the process of thermoinactivation the ions of the euglobulin compound combine or interact chemically with substances contained in the pseudoglobulin and albumin fraction.

The protection against destruction afforded by the presence of NaCl on the alkaline side of pH 6.1 to 6.4 may be explained by the depression in ionization of the Na euglobulin compound existing

[16] Rona, P., and Michaelis, L., *Biochem Z.*, 1910, xxviii, 193.
[17] Coulter, C. B., *J. Gen. Physiol.* 1920–21, iii, 309.
[18] Unpublished experiments.
[19] Personal suggestion.

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at these reactions[20] which is caused by the high concentration of the common Na ion. On the acid side of pH 6.1 to 6.4 the protein exists in amounts increasing with the acidity in the cationic condition. We should expect a similar depression in the ionization of the protein in this form to be caused by the excess of the common Cl ion, but since at these reactions the destruction of complement is as great in the presence as in the absence of salt it is apparent that the behavior of the protein as cation is different from that as anion. This conclusion was suggested by Brooks[21] from a somewhat different evaluation of similar data.

CONCLUSIONS.

1. The destruction which complement undergoes on being heated in dilution in distilled water is least at a reaction between pH 6.1 and 6.4. This depends upon the relative preservation of the mid-piece function at this point. This reaction represents probably the isoelectric point of a compound of the euglobulin with some substance present also in serum.

2. During the process of thermoinactivation it is chiefly or entirely the ions of this euglobulin compound which react, and these combine or interact with substances contained in the pseudoglobulin and albumin fraction.

3. The behavior of the euglobulin is different in the anionic and in the cationic condition, since on the acid side of pH 6.1 to 6.4 the destruction by heat increases as rapidly with the acidity in the presence as in the absence of NaCl. On the alkaline side of this point the presence of NaCl protects complement from destruction because of the depression in the ionization of the euglobulin.

[20] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

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A SIMPLE CASE OF SALT ANTAGONISM IN STARFISH EGGS.

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The freshly laid egg of the common starfish, *Asterias forbesi*, is surrounded by a thin layer of water-swollen or gelatinous material, of about 15 to 20 microns in diameter (one-eighth to one-twelfth the egg diameter of about 160 microns), invisible to ordinary observation, but readily demonstrated by mounting the eggs in a somewhat thick suspension of India ink in sea water and examining on a slide under a moderate magnification. Each egg then appears surrounded by a clear halo outlining the space between the egg surface and the outer limit of the jelly which is impermeable to the ink particles. It is present in all full sized eggs, both mature and immature, but is absent in the smaller ovarian eggs of two-thirds or less the full diameter; evidently the water-swollen material of which it consists is separated from the egg during the later period of ovarian growth. In the starfish egg the jelly is remarkably resistant to solution in ordinary sea water and is not removed by repeated washing in this medium, even with the aid of centrifuging; in this respect the *Asterias* egg differs from the *Arbacia* egg which is surrounded by a jelly otherwise similar but readily removed by washing in sea water. In unfertilized starfish eggs the jelly shows no change after 24 hours in sea water; but it gradually disappears in fertilized cleaving eggs, possibly in consequence of increased separation of CO_2 from the eggs, since, as Garrey[1] has shown, it combines with acids and in so doing acquires increased swelling properties.

[1] Garrey, W. E., *Biol. Bull.*, 1919, xxxvii, 287.

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The jelly is readily and rapidly removed from the eggs by washing in pure isotonic NaCl solution (0.54 M). This is most conveniently demonstrated as follows. Sea water containing a suspension of the eggs is gently centrifuged with a hand centrifuge, with ten or twelve slow turns, so as to collect the eggs in the narrow end of the tube; the sea water is poured off and replaced by pure 0.54 M NaCl; the tube is then inverted to suspend the eggs and again slowly centrifuged as before; the solution is poured off and replaced with fresh, and this process is repeated. The eggs are thus exposed to the pure NaCl solution free from all but traces of sea water. If they are then returned to sea water and examined in India ink, the jelly is found to have completely disappeared. There is no evidence of further secretion of jelly by eggs which have thus been freed from it and returned to sea water.

Removal of the jelly leaves the power of fertilization and development unimpaired. Thus, in a typical experiment unfertilized eggs, removed from the animal 45 minutes previously, were exposed for 6 minutes to 0.54 M NaCl with two changes of the solution and centrifuging as above. Examination in India ink showed complete removal of the jelly from all eggs. Sperm was added to part of the eggs 18 minutes after the return to sea water. A majority next day had formed blastulæ; abnormalities were, however, more frequent than in the untreated control eggs and about 20 per cent had died without development; this injurious effect is to be referred to the action of the pure unbalanced solution upon the eggs. Similarly, eggs freed from jelly subsequently to fertilization continued development and formed blastulæ, although showing also a larger proportion of abnormalities than the control.

Arbacia eggs treated as above with 0.54 M NaCl show a similar disappearance of the jelly, but the same effect is seen in sea water and in solutions of NaCl containing $CaCl_2$. In this species the jelly is more soluble than in *Asterias* and the difference between pure and calcium-containing NaCl solutions is not shown by the above method. This difference in the properties of the jelly layer in the two species indicates a specific difference of chemical composition, but so far I have made no attempt to investigate this difference in detail.

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In both *Asterias* and *Arbacia* exposure of the eggs to the pure NaCl solution for a few minutes alters the consistency of the cell surface, and apparently also the permeability of the plasma membrane, in a highly characteristic manner. The eggs cohere in small clumps, or agglutinate; this effect is well marked even in eggs exposed to the pure NaCl solution in a finger-bowl without any centrifuging, but the latter process promotes the formation of larger and firmer aggregates. This agglutination, although accompanying the removal of the jelly, is an entirely independent process, and like the removal of the jelly is prevented by the presence of calcium in the solution. A further characteristic effect produced by the pure NaCl solution in *Asterias* eggs (but not in *Arbacia*) is the formation of apparently normal fertilization membranes in a certain proportion of eggs; and in some cases cleavage and development to a blastula stage result.[2] This membrane-forming and activating effect is also antagonized by calcium.[3]

The solution of the jelly in pure NaCl solution is a somewhat gradual process; the jelly gradually incorporates water, and swells, eventually losing coherence and passing into solution. The process of swelling may be arrested at any stage by returning to sea water; but there is no evidence of reversal in the sense of a return of the jelly to its original water content. Eggs were placed in a finger-bowl, the sea water removed as far as possible, and to the remaining mass of eggs (about 1 cc.) 100 cc. of 0.54 NaCl were added. From the NaCl solution eggs were returned to finger-bowls containing sea

[2] Lillie, R. S., *Am. J. Physiol.*, 1910, xxvi, 106; cf. 119.

[3] Lillie, R. S., *Am. J. Physiol.*, 1910–11, xxvii, 289. A further interesting effect of the pure isotonic NaCl solution, also antagonized by $CaCl_2$, is that it prevents the dissolution of the germinal vesicle, and hence the maturation process, in starfish eggs placed in the solution immediately after removal from the animal. If such eggs are left in the pure NaCl solution for from 5 to 10 minutes, and are then returned to sea water, they remain permanently immature. Similar treatment with a calcium-containing NaCl solution (*e.g.*, 95 volumes of M/2 NaCl plus 5 volumes of M/2 $CaCl_2$) leaves them apparently unaffected. The pure NaCl solution thus produces the same effect as weak fatty acid solution or high temperature, either of which, if applied at this time, also prevents maturation (cf. Lillie, R. S., *Biol. Bull.*, 1917, xxxii, 135). Mineral acids appear to have a similar effect (cf. Loeb, J., *Arch. ges. Physiol.*, 1902, xciii, 59).

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water at the following intervals: ¼, ½, 1, 2, 3, 4, 6, 8, and 12 minutes; after 20 to 30 minutes in sea water, eggs from each bowl of the series were examined on a slide in India ink suspension. It was found that the jelly still remained about eggs that had been exposed to the NaCl solution for so long as 3 or 4 minutes, but that by that time it was swollen to several times its original volume, so that the boundary of the ink suspension was often separated from the egg by a full egg diameter. In eggs exposed to NaCl solution for 6 minutes and examined under the same conditions the jelly had completely disappeared from a large proportion of eggs, though still remaining in a greatly swollen form in most. After 12 minutes exposure the ink was in nearly all cases in contact with the egg surface, indicating complete removal of the jelly.

The removal of the jelly in NaCl solution is completely prevented by the addition of a small proportion of $CaCl_2$. The agglutinating and membrane-forming effects above described are at the same time prevented or greatly decreased. Since these two latter effects are dependent upon alteration of the surface layer of the living protoplasm, this parallelism suggests that the calcium prevents the physiological action of the pure NaCl solution through some characteristic influence on the solubility or hydration of certain compounds which are present in the protoplasmic surface layer and upon which the normal properties of this layer depend.

The following typical experiment (June 18, 1920) will illustrate. Starfish eggs were removed from the animal at 3.06 p.m.; the eggs were normal; all showed the typical jelly envelope in India ink suspension; more than 90 per cent underwent maturation and upon fertilization developed to larval stages. At 3.48 p.m. the two tubes of a hand centrifuge were filled with a suspension of unfertilized eggs in sea water and gently centrifuged until the eggs were settled in a mass about one-third of an inch deep in the narrow part of each tube. The sea water was then poured off and replaced in one tube by pure 0.54 M NaCl (lot A), in the other by a mixture of 250 cc. 0.54 M NaCl plus 15 cc. M/2 $CaCl_2$ (lot B). The solutions were added at 3.49 p.m.; the tubes were then gently centrifuged to settle the eggs and the solution was changed; this procedure was repeated. The eggs were then returned to sea water. The total time

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of exposure to the solutions was 6 minutes. Examination in India ink suspension about 15 minutes later showed complete removal of the jelly in the eggs of lot A, of which about 25 to 30 per cent had also formed fertilization membranes. In the eggs of lot B the jelly was unaltered and no fertilization membranes were formed. Part of the eggs of both lots were fertilized with sperm at 4.13 p.m.; the next day about 70 per cent of the eggs of lot A had formed blastulæ, largely abnormal, while almost all of lot B had developed normally.

Other experiments of the same kind give similar results. Exposure to pure NaCl solution for some minutes removes the jelly; it also causes agglutination and injures the eggs, as shown by a certain degree of impairment and abnormality in development, and forms fertilization membranes in a considerable proportion of eggs. All of these effects are prevented by the addition of a little $CaCl_2$ to the solution.

The antagonistic action of calcium is shown in much more dilute solution than the above; *e.g.*, in M/400 $CaCl_2$ (199 volumes of 0.54 M NaCl plus 1 volume of M/2 $CaCl_2$), with the same treatment as above, the jelly remained intact. Further experiments to determine the limit of the effective concentrations were not tried. Magnesium chloride has a similar action in preventing the solution of the jelly, but is less effective.

The question of whether calcium can be replaced in this relation by other polyvalent metals has not yet been experimentally tested except in the case of aluminium. Eggs were placed in pure 0.54 M NaCl (lot A), and in 99 volumes of 0.54 M NaCl plus 1 volume of M/2 $AlCl_3$ (lot B); the solutions were changed twice by centrifuging and decanting as before. On return to sea water after 6 minutes in the solutions the eggs of lot A were found free from jelly and largely agglutinated, while those of lot B retained the jelly, although this was somewhat thinner than in the untreated control eggs; there was no agglutination. The probability is that many other polyvalent cations would be found to have this effect, as in other cases of antagonism.

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THEORY.

In the present case of salt antagonism the effect, removal of the jelly, is a direct result of the incorporation of water in pure NaCl solution with consequent swelling until coherence is lost; in the same solution containing a little calcium the jelly retains its normal hydration and remains coherent and water-insoluble. The chemical composition of the jelly is unknown, but the presence of a certain proportion of protein is probable; this is suggested by its acid-combining properties,[1] also by its mode of formation as a cellular secretion, its physical consistency and its variation in properties from species to species. At least it seems clear that some substance is present forming compounds (salts) with sodium and calcium, which differ in their solubilities and in their affinities for water.

Apparently, in the presence of an excess of NaCl, a Na salt with a marked affinity for water is formed; hence the swelling and eventual solution. When $CaCl_2$ is present in the solution a water-insoluble Ca salt is formed; in the presence of a certain proportion of this the jelly as a whole remains insoluble. In other words, the normal coherence and water-insolubility of the jelly depend on the presence of a water-insoluble compound which pervades the system and renders it coherent and water-insoluble. Hence the removal of this by replacement of Ca with Na renders the whole structure soluble.

Antagonisms depending on differences in the solubilities of Na salts (or in general of alkali metal) and Ca salts (or alkali earth and heavy metal) are well known in purely physical systems. In many organic acids the Na salts are highly soluble in water, while the Ca salts are insoluble. The case of soaps is probably the most relevant to the conditions in biological systems. The physicochemical antagonisms recently investigated by Clowes,[4] using salt solutions which were allowed to drop from a stalagmometer through oil containing some fatty acid, depend on the differences between the solubilities of Na soaps and Ca soaps in the oil and the water phases respectively. The antagonisms between the influence of Na and Ca salts on the precipitation of lecithin[5] and heat-denatured egg white[6] are prob-

[4] Clowes, G. H. A., *J. Physical Chem.*, 1916, xx, 407.
[5] Koch, W., *Z. physiol. Chem.*, 1909, lxiii, 432.
[6] Mathews, A. P., *Am. J. Physiol.*, 1905, xiv, 203.

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ably in part determined by similar conditions, although factors peculiar to suspensoid systems no doubt also enter in these cases. The properties of soaps, which are at once soluble in water and in many organic solvents, and which are consequently highly surface-active at water lipin interfaces, are probably of fundamental importance in protoplasmic activities. It is noteworthy that the water-combining properties of tissues and cells are influenced by salts in a manner consistent with the hypothesis that soaps or compounds with similar solubilities determine the manner in which the bound water (Overton's "Quellungswasser")[7] is held in the protoplasm. Many years ago Loeb[8] called attention to the parallel between the absorption of water by muscle immersed in isotonic solutions of different salts of alkali and alkali earth metals, and by the soaps of the same metals; thus, the muscle absorbed much more water in isotonic KCl solution than in NaCl, while in isotonic $CaCl_2$ it lost water; when the corresponding soaps are immersed in water a similar order of relative absorption is seen, K soaps (soft soaps) swelling more rapidly than Na soaps, while the water-insoluble Ca soaps do not take up water. Swelling and loss of consistency or turgor in plant tissues immersed in pure NaCl solution are well known phenomena, which have recently been investigated in great detail by Hansteen;[9] the disintegration and loosening of the intercellular coherence which he describes as occurring under these conditions are prevented by the presence of small quantities of calcium salts. Hansteen calls attention to the earlier work of Mangin[10] on the rôle of the pectin compounds in the interstitial substance or middle lamella of plant tissues; the cementing properties of this layer are, according to Mangin, dependent on the presence of an insoluble Ca compound which he calls "Ca pectinate." In the presence of NaCl solution free from Ca this layer becomes soluble and absorbs water and the cells fall apart. Herbst's[11] observations on the loss of coherence of of the blastomeres of sea-urchin eggs in Ca-free sea water probably

[7] Overton, E., *Arch. ges. Physiol.*, 1902, xcii, 115.
[8] Loeb, J., *Arch. ges. Physiol.*, 1899, lxxv, 303.
[9] Hansteen, B., *Jahrb. wiss. Bot.*, 1910, xlvii, 289; 1913–14, liii, 536.
[10] Mangin, L., *J. Bot.*, 1893 (cited from Hansteen).
[11] Herbst, C., *Arch. Entwcklngsmechn.*, 1900, ix, 424.

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have a similar general significance. The coherence of the ciliated cells in the gill epithelium of mollusca (Mitylus) is similarly lost in pure isotonic solutions of many Na salts, in which also the cells absorb large quantities of water;[12] these effects are prevented by the addition of Ca to the solution. The action of pure NaCl solutions in increasing the permeability of plant tissues to ions,[13] and of structures like the membrane of the *Fundulus* egg[14] to salts and water, is closely related to the above; similarly with the breakdown of protoplasmic structures like cilia and plasma membranes in this solution;[15] here the structural continuity is lost and with it the dependent properties of coherence and semipermeability. In all of these cases an essential part of the protective or antitoxic action of the calcium consists in preventing physical disintegrations of this kind; such disintegrations are apparently the direct result of replacing solid water-insoluble material and structure by water-soluble.

The difference between the water-combining powers of Na and Ca proteinates has been pointed out recently by Loeb in a series of papers on the influence of inorganic salts on the physical properties of proteins;[16] here also characteristic salt antagonisms affecting physical properties such as viscosity, swelling, osmotic pressure, precipitability by alcohol, are observed when the Na and Ca salts are present in certain proportions.[17] It seems probable that certain types of biological salt-antagonisms are to be explained by reference to general facts of this kind; the case of the *Fundulus* egg, where the toxic action of the pure NaCl solution is associated with a destruction of the water-proof character of the membrane or chorion enclosing the egg,[14] appears to exemplify this condition. In the case of antagonisms in living protoplasm, however, not only the proteins but other compounds, and especially the lipoids, appear to be essentially concerned. The salt antagonisms studied by Clowes,[4] which

[12] Lillie, R. S., *Am. J. Physiol.*, 1906–07, xvii, 89.

[13] Osterhout, W. J. V., *Science*, 1912, xxxv, 112; xxxvi, 350.

[14] Loeb, J., *Science*, 1912, xxxvi, 637; *Biochem. Z.*, 1912, xlvii, 127.

[15] Lillie, R. S., *Am. J. Physiol.*, 1903–04, x, 419; 1909, xxiv, 23; 1912–13, xxxi, 259.

[16] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; 1918, xxxiv, 77.

[17] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 3; 1918, xxxiv, 395; 489; and xxxv, 497.

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exhibit such a remarkable parallelism with physiological antagonisms, are referable to the formation of soaps or soap-like compounds. It seems probable that variations in the partition of such compounds between the aqueous and the non-aqueous or organic solvents of the protoplasm are important factors in the physiological effects produced by varying the proportion of the inorganic salts in the medium. Clowes attributes the variations in the drop numbers of the solutions flowing through the oil from the stalagmometer to variations in the ratio of the oil-soluble to the water-souble soaps. These soaps have opposite influences on the surface tension factor opposing the detachment of the drop. Oil-soluble compounds like higher alcohols influence the drop numbers in the same manner as Ca salts. The recent observations of Heilbrunn[18] on changes in protoplasmic consistency under the influence of lipoid-solvent compounds also suggest that variations in the partition of compounds between the aqueous and the lipoid phases of protoplasm may influence greatly the type of protoplasmic consistency or structure.

In general, it may be inferred from the above facts that the presence of a certain proportion of solid water-insoluble salt-like compounds in the protoplasmic surface film is necessary to the normal semi-permeability and structural permanence of this structure; similar considerations apply to filamentous solid structures like cilia and to the other solid structural elements of the cell. This view explains the destructive action of pure solutions of alkali salts like NaCl, which have the effect of substituting water-soluble Na-compounds for the water-insoluble Ca compounds (*e.g.*, soaps) normally present. Hansteen's[9] results with plant tissues afford strong evidence that water-insoluble Ca compounds are essential to the stability of the normal protoplasmic structures, and also of cell walls and similar structures formed by protoplasmic activity. He attributes great importance to lipoid compounds present in the protoplasmic surface layers and in the cell walls, and especially to the formation of water-insoluble combinations between these materials (together with pectin) and calcium. He regards these calcium compounds as necessary for the normal coherence of cells, as shown by their presence

[18] Heilbrunn, L., *Biol. Bull.*, 1920, xxxix, 307.

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in the middle lamella, and also for the normal properties of the surface protoplasm.[19] According to Hansteen, pure alkali salt solutions attack and alter primarily the lipoid constituents of the cell walls,[20] inducing absorption of water and consequent disintegration; and he cites especially the work of Krefting,[20] who obtained from the intercellular substance of brown algæ an acid material ("Tangsäure") which forms water-soluble salts with alkali metals and water-insoluble salts with alkali earths (Ca, Sr, Ba). This compound apparently corresponds with the Ca pectinate to which Mangin attributed the cohesive properties of the material composing the middle lamella.

Hansteen's further observation that the presence of Ca salts in culture solutions is highly favorable to the branching of the roots and the growth of root-hairs in seedlings also indicates that Ca compounds are necessary to the formation of the solid structures essential to normal growth. For example, the development of root-hairs is greatly promoted in media containing several times the normal concentration of Ca salts, a result which has recently been confirmed by Wiechmann[21] in Höber's laboratory. Wiechmann finds also that strontium, barium and certain heavy metals (Mn, Ni, Co) act similarly to calcium, while magnesium is ineffective. Such data, when considered in relation to those cited above, throw an interesting light on the general significance of calcium in the formation of organic structure. Certain properties of the living protoplasmic structures, such as rigidity, water-insolubility and impermeability to water-soluble substances, seem to require the presence of calcium compounds.[22] The rapid alteration of the superficial protoplasmic

[19] Hansteen, B., *Jahrb. wiss. Bot.*, 1910, xlvii, 374.

[20] Hansteen, B., *Jahrb. wiss. Bot.*, 1913–14, liii, 574.

[21] Wiechmann, E., *Arch. ges. Physiol.*, 1920, clxxxii, 99.

[22] The general view that the presence of water-insoluble materials in the plasma membranes (formed from inorganic salts present) is responsible for their peculiar osmotic properties is by no means a new one, having apparently first been suggested by Traube in 1867 on the basis of his work with precipitation membranes. Recently the subject has been reviewed by Meigs (Meigs, E. B., *Am. J. Physiol.*, 1915, xxxviii, 456), who has studied the effects of impregnating colloidal membranes with various inorganic precipitates. He finds that membranes made by precipitating Ca and Mg phosphates in thin sheets of celloidin show

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layer or plasma membrane in pure solutions of Na salts is of a kind which is consistent with this interpretation, since the essential feature of the effect produced is an increase of permeability, allowing the ready penetration of water-soluble substances (sugars, neutral salts, etc.) to which previously the membrane formed a complete barrier.[22]

SUMMARY.

The jelly surrounding the eggs of the starfish, *Asterias forbesi*, is insoluble in normal sea water, but rapidly swells and dissolves when the eggs are washed in a pure isotonic solution of NaCl. In the presence of a small proportion of $CaCl_2$ this solvent and disintegrative action of the NaCl solution is entirely prevented, and in the mixed solution the jelly exhibits the same insolubility and other properties as in normal sea water.

2. This action of $CaCl_2$ in preventing the dissolution of the jelly runs parallel with its action in preventing certain definite effects

many resemblances to plasma membranes in their osmotic properties, especially in their slight permeability to soluble inorganic salts and sugar, and suggests that these phosphates may play a part in determining the properties of the membranes of cells. This possibility should not be overlooked, although the above evidence indicates that insoluble salts formed with the colloidal compounds of the cell are of chief importance.

[22] Apparently the biologically essential properties of an organic membrane are referable chiefly to those of the limiting surface layers. Hence the cases of salt antagonisms at metallic and other surfaces have also an intimate bearing on the present problem. Bredig and Weinmayr (*Z. physik. Chem.*, 1903, xlii, 601) cite cases of such influence in the Hg–H_2O_2 catalysis; thus KOH and KCl may act as antagonists; the rhythm is extinguished by adding KCl and is restored by KOH. Related phenomena are seen in the influence of cations of high oxidation potential, like Ag, in preventing the spontaneous activation of passive iron in solutions of $NaNO_3$ and similar salts (Lillie, R. S., *Science*, 1919, 1, 416). Evidently the permeability and electromotor properties of any interfacial film depend on its composition and physical condition, both of which vary with the internal composition of the two adjoining phases. Both physical and chemical factors are concerned in the formation of the plasma membrane; adsorption, by which (presumably) the surface materials are assembled, is usually classed as a physical process; but the special composition of the membrane depends on the specific character of the cell metabolism, and on the nature of the reactions (oxidations, etc.), occurring at the cell surface.

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of the pure NaCl solution on the living egg (agglutination, cytolytic action, membrane formation, prevention of maturation).

3. The inference is that the essential factor in these and similar antagonistic and protective actions is the formation of solid water-insoluble colloidal salts (*e.g.*, soaps and proteinates) of calcium (or other metal) with the structural colloids of the protoplasm. Apparently the presence of a certain proportion of such compounds is necessary to the structural stability of the living protoplasm, and especially to the water-insolubility and semipermeability of its external layer or plasma membrane. When the cell is immersed in the pure NaCl solution, water-soluble Na compounds are substituted for the insoluble Ca compounds which normally provide the necessary insolubility and coherence, and disintegration results.

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STUDIES IN WOOD DECAY.

II. Enzyme Action in Polyporus Volvatus Peck and Fomes Igniarius (L.) Gillet.

By HENRY SCHMITZ.

(From the Laboratory of Forest Products, School of Forestry, University of Idaho, Moscow.)

(Received for publication, April 25, 1921.)

The present paper is the third of a series,[1,2] of papers dealing with the enzyme action in the wood-destroying fungi. It is the second of a series[3] of papers to be issued by this Laboratory dealing with the decay of wood in the broadest sense.

From the standpoint of parasitism, *Polyporus volvatus* is one of the most interesting of the wood-destroying fungi. Although no inoculation experiments have been made, numerous observations tend to confirm the opinion of the writer that *Polyporus volvatus* is truly parasitic. Throughout Washington, Oregon, and Idaho it is not at all unusual to find fruiting bodies of *Polyporus volvatus* appearing in great numbers over practically the entire surface of the trunk of Douglas fir, white fir, and western hemlock. This condition may be observed on trees still having a green, healthy foliage as well as on trees which to all appearances have been killed by the fungus.

[1] Schmitz, H., and Zeller, S. M., Studies in the physiology of the fungi IX. Enzyme action in *Armillaria mellea* Vahl., *Daedalea confragosa* (Bolt.) Fr., and *Polyporus lucidus* (Leys.) Fr., *Ann. Missouri Bot. Garden*, 1919, vi, 193–200.

[2] Schmitz, H., Enzyme action in *Echinodontium tinctorium* Ellis and Everhardt, *J. Gen. Physiol.*, 1919–20, ii, 613.

[3] Schmitz, H., and Daniels, A. S., Studies in wood decay I. Laboratory tests on the relative durability of some western coniferous woods with particular reference to those growing in Idaho, School of Forestry, Univ. Idaho, Bull. 1, 1921, 1–12.

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The fact that *Polyporus volvatus* may be parasitic seems to have been first suggested by Zeller[4] in an unpublished paper. Dr. Zeller writes in part, "The fact that there were still needles on the last year's growth and that the mycelium had spread from the base of the tree up the trunk for 45 to 50 feet indicates that the fungus is parasitic. The writer has no absolute proof of this statement."

In this region *Fomes igniarius* is the cause of a serious white heart rot in the common aspen (*Populus tremuloides*) and also causes a a similar heart rot in mountain birch (*Betula fontinalis*). Quite recently Weir[5] has reduced *Fomes igniarius* and *Fomes nigricans* Fr. to synonomy.

Thus, the two fungi considered here are not without considerable interest both from the general and economic point of view.

Methods.

The cultures of the fungi used in the present study were obtained from young sporophores by the tissue method. As before, the cultures were grown on sterile carrots and while still in an active growing condition, removed from the flasks, dried, and ground. All of the enzyme cultures were set up in duplicate and 0.25 gm. of fungous meal was invariably used. Otherwise, the methods followed are those previously described.

Esterases.

The esterase activity of *Polyporus volvatus* and *Fomes igniarius* was studied by the use of 1 per cent solutions of methyl acetate, ethyl acetate, ethyl butyrate, triacetin, and olive oil emulsion. After twenty-one days incubation, hydrogen ion concentration determinations were made of the various filtrates. Positive esterase activity was obtained in the case of both fungi when methyl acetate was used as the substrate. The action on all the other substrates was negative.

[4] Zeller, S. M., Wood destroying fungi of Washington. Unpublished paper.

[5] Weir, J. R., Some observations on abortive sporophores of wood destroying fungi. *Phytopathology*, 1915, v, 48–50.

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Carbohydrases.

The action of carbohydrases was determined on 1 per cent solutions of maltose, lactose, sucrose, raffinose, potato starch, inulin, white fir cellulose, and hemicellulose from date seed endosperms. After varying periods of incubation, the cultures were filtered and 5 cc. samples of the filtrates treated with 20 cc. of Fehling's solution. The results in the following table are the average of two titrations and are expressed as the number of cubic centimeters of 0.05 N potassium permanganate solution required to oxidize the dissolved copper.

TABLE I.

Carbohydrase Action of Polyporus volvatus and Fomes igniarius.

Incubation period.	Substrate.	*Polyporus volvatus.*			*Fomes igniarius.*	
		With fungous meal.	With fungous meal autoclaved.	Without fungous meal.	With fungous meal.	With fungous meal autoclaved.
		0.05 N $KMnO_4$				
days		*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
14	Maltose	21.85	14.70	13.25	30.40	17.15
20	Lactose	26.65	21.40	18.30	29.60	22.00
3	Sucrose	4.35	3.65	0.75	13.10	6.00
4	Raffinose	8.80	3.55	0.10	11.50	4.35
4	Potato starch	19.00	2.75	0.10	8.10	3.55
10	Inulin	5.00	3.10	0.15	7.50	5.00
30	White fir cellulose	6.00	2.85	0.20	8.00	4.95
30	Hemicellulose	7.80	3.45	1.80	8.70	5.25

Glucosidase.

The presence of a glucosidase in the fungous meal of the two fungi here discussed was determined by the action of the fungous meal upon a 1 per cent solution of salicin. After a 5 day incubation period, the enzyme cultures were filtered and sugar determinations made of 5 cubic centimeter samples of the filtrates. The results, expressed in the number of cubic centimeters of 0.05 N potassium permanganate solution required to oxidize the dissolved copper are shown in Table II.

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Tannase.

Under the action of tannase, digallic acid is probably converted into gallic acid in accordance with the general formula,

$$C_{14}H_{10}O_9 - H_2O = 2C_6H_2(OH)_3COOH$$

Whether or not this reaction took place was determined by titrating the filtrates against 0.05 N iodine after the digallic acid had been precipitated out by the addition of albumin and the albumin salted out with NaCl. As indicated in Table III, the results are negative.

TABLE II.

Glucosidase Action of Polyporus volvatus and Fomes igniarius.

	Polyporus volvatus.	*Fomes igniarius.*
	0.05 N $KMnO_4$	
	cc.	cc.
1 per cent salicin + fungous meal	11.10	15.15
1 " " " + " " autoclaved	3.50	4.50
1 " " "	0.40	0.40

TABLE III.

Tannase Action of Polyporus vovatus and Fomes igniarius.

Enzyme Culture.	*Polyporus volvatus.*	*Fomes igniarius.*
	0.05 N iodine.	
	cc.	cc.
1 per cent digallic acid + fungous meal	4.7	3.9
1 " " " " + " " autoclaved	4.7	3.9
1 " " " "	3.5	3.5

The absence of tannase in *Polyporus volvatus* is difficult to account for since the fungus inhabits the barks of trees having a high tannin content. Its absence may, however, be due to the fact that the culture medium upon which it had been grown contained little or no tannin. The influence of the culture medium on the production of tannase in fungi has been fully investigated by Knudson.[6]

[6] Knudson, L., Tannic acid fermentation. I, *J. Biol. Chem.*, 1913, xiv, 159, Figs. 1 and 2; Tannic acid fermentation. II, 185.

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Urease and Amidase.

To determine the presence or absence of enzymes which split amino-acids and urea into ammonia and hydroxy acids, the indicator method suggested by Schmitz and Zeller[1] was employed. The results are shown in Table IV.

TABLE IV.

Urease and Amidase Activity of Polyporus volvatus and Fomes igniarius.

	Enzyme culture.		Approximate change in hydrogen ion concentration.	
			Urea.	Acetamid.
Polyporus volvatus.	Substrate + fungous meal.	1	*pH* 5.6–6.0 3 min.	5.6–5.6 3 min.
		2	5.6–6.0 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal autoclaved.	3	5.6–6.0 3 min.	5.6–5.6 3 min.
		4	5.6–6.0 3 min.	5.6–5.6 3 min.
Fomes igniarius.	Substrate + fungous meal.	1	5.6–7.8 45 sec.	5.6–5.6 3 min.
		2	5.6–7.8 45 sec.	5.6–5.6 3 min.
	Substrate + fungous meal autoclaved.	3	5.6–6.0 3 min.	5.6–5.6 3 min.
		4	5.6–6.0 3 min.	5.6–5.6 3 min.
Control.	Substrate alone.	1	5.6–6.0 3 min.	5.6–5.6 3 min.
		2	5.6–6.0 3 min.	5.6–5.6 3 min.

Positive results were obtained only in the case of *Fomes igniarius* when urea was used as a substrate.

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Rennet.

When 0.25 gm. of fungous meal was added to 10 cc. of fresh milk, coagulation occured in 3 hours in the case of *Polyporus volvatus*, and in 4½ hours in the case of *Fomes igniarius*.

Catalase.

The presence of catalase was demonstrated by the action of the fungous meal upon a 3 per cent solution of hydrogen peroxide. When 0.25 gm. of the meal was added to 100 cc. of solution, 9.5 cc. of oxygen were liberated in the case of *Polyporus volvatus*, and 12 cc. in the case of *Fomes igniarius* in a period of 5 minutes.

Protease.

Tryptic and ereptic fermentation was studied by the use of albumin, peptone, casein, and fibrin in enzyme cultures having a neutral acid and alkaline reaction. The biuret test was employed in testing for the action of erepsin. The enzyme cultures were filtered, crystalline ammonium sulphate was added to the filtrate to precipitate the native proteins, and the solutions were again filtered through bone-black to remove the precipitate. In no case was a pink color produced when sodium hydroxide and dilute copper sulphate were added to the second filtrate.

A tryptophane test was also made of the filtrate by adding a few drops of glacial acetic acid and a few drops of strong bromine water. In no case was a pink color produced.

SUMMARY.

Circumstantial evidence is presented which indicates that *Polyporus volvatus* is parasitic.

Cultures of *Polyporus volvatus* and *Fomes igniarius* may be obtained from the young sporophores by the tissue method.

In *Polyporus volvatus* the presence of the following enzymes was demonstrated: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, glucosidase, rennet, and catalase.

In *Fomes igniarius* the presence of the following enzymes was demonstrated: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, glucosidase, urease, rennet, and catalase.

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STUDIES ON THE REGULATION OF OSMOTIC PRESSURE.

I. The Effect of Increasing Concentrations of Gelatin on the Conductivity of a Sodium Chloride Solution.

By WALTER W. PALMER, DANA W. ATCHLEY, and ROBERT F. LOEB.

(From the Chemical Division of the Medical Clinic of the Johns Hopkins University and Hospital.)

(Received for publication, May 20, 1921.)

INTRODUCTION.

In a study of the factors influencing the regulation of osmotic pressure of the body fluids, the physical properties have been determined in a series of blood sera from normal and pathological individuals. In the case of some patients, there was found a strikingly low percentage of serum protein. The conductivity of the salts in blood with a low percentage of serum protein was high compared with the conductivity of the same salts in normal blood. It occurred to us that possibly the protein molecules offer mechanical interference to the passage of the current through the solution, hence, when the protein content of the solution is diminished the observed conductivity will increase without a corresponding increase in the concentration of ions present. It was to throw light upon this hypothesis that a study of the relation between conductivity and concentration of gelatin was undertaken.

In 1898 Bugarszky and Tangl[1] pointed out that, of the non-conducting substances in the serum, protein is the only one present in amounts sufficient to affect the electrical conductivity. These authors derived a formula by the addition of serum protein in varying quantities to a salt solution of constant conductivity. The serum protein was obtained by dialyzing blood serum for two months and concentrating under reduced pressure. The hydrogen ion concentration was not controlled in their experiments.

[1] Bugarszky, St., and Tangl, F., *Arch. ges. Physiol.*, 1898, lxxii, 531.

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EXPERIMENTAL.

Powdered gelatin was used in these experiments and was purified at the isoelectric point according to the method described by Loeb.[2] The percentage of gelatin in the final solutions was determined by drying samples to constant weight. The hydrogen ion concentration was determined by a gas chain and the conductivity measurements were made with a Kohlrausch bridge at 25°C. The physical methods were standardized by simple inorganic solutions. Duplicate observations were made in each case. The concentrations of gelatin ranged from 0.8 per cent to 6.5 per cent and the experiments were carried out at three different hydrogen ion concentrations. One series of observations was made at pH 5.1, near the isoelectric point of gelatin,

TABLE I.

Conductivity of Pure Gelatin Solutions.

Gelatin.	Conductivity $\times 10^{-4}$ at pH 3.3	Conductivity $\times 10^{-4}$ at pH 5.1	Conductivity $\times 10^{-4}$ at pH 7.4
per cent			
0.8	6.6	0.3	1.3
1.7	10.7	0.6	2.4
3.3	19.1	1.0	4.2
4.9	25.7	1.3	5.9
6.5	30.9	1.5	7.2

another at 3.3 because of the relatively high ionization of gelatin at that acidity, and finally one at pH 7.4, approximately the reaction of blood. The gelatin was brought to these hydrogen ion concentrations by the addition of HCl or NaOH.

A preliminary experiment, Table I, was carried out on pure gelatin solutions varying in concentration from 0.8 per cent to 6.5 per cent of gelatin. The conductivity of these solutions was determined at the three different hydrogen ion concentrations mentioned above, *viz.* 5.1, 7.4, and 3.3. Fig. 1 shows the concentrations of gelatin as the abscissæ and the specific conductivities $\times 10^{-4}$ as ordinates. The curves at the three reactions are reduced to the same scale and plotted at equal intervals above each other. The conductivity in-

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 341.

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creases with each increment of gelatin added but the curve is not a straight line in any instance. The actual change in conductivity as the concentration of gelatin increases from 0.8 per cent to 6.5 per

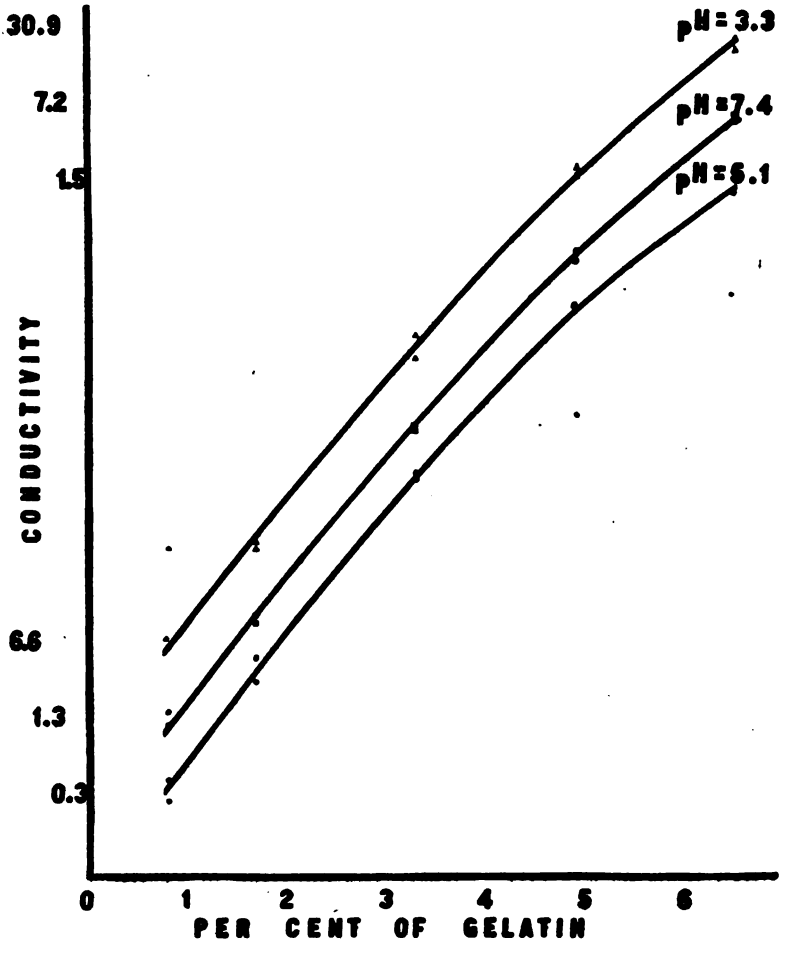

FIG. 1. Conductivities of pure gelatin solutions. The abscissæ represent varying concentrations of gelatin in per cent and the ordinates represent specific conductivities $\times 10^{-4}$. With increase in concentration of gelatin from 0.8 per cent to 6.5 per cent it is seen that the conductivity of gelatin at pH 5.1 increases from 0.3 to 1.5, at pH 7.4 it increases from 1.3 to 7.2 and at pH 3.3 it increases from 6.6 to 30.9.

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cent is from 0.3 to 1.5 at pH 5.1 while at 3.3 the range is from 6.6 to 30.9. This difference in ionization with change in hydrogen ion concentration has been thoroughly studied in 1 per cent gelatin solutions by Loeb.[3]

The next series of experiments, Table II, bears more directly upon the original problem and consists of observations on the effect of gelatin upon the conductivity of a 0.6 per cent sodium chloride solution. The concentrations of gelatin and the reactions of the final solutions were the same as were used in the first experiments. Fig. 2 shows the results plotted on a common scale, conductivities again as ordinates and concentrations of gelatin in per cent as abscissæ. The curves for each pH are appropriately labeled. All three curves

TABLE II.

Conductivity of 0.6 per cent NaCl Solutions with Increasing Gelatin.

Gelatin.	Conductivity × 10^{-4} at pH 3.3	Conductivity × 10^{-4} at pH 5.1	Conductivity × 10^{-4} at pH 7.4
per cent			
0.8	114.5	108.9	109.8
1.7	115.2	106.7	108.2
3.3	117.3	102.6	105.9
4.9	119.8	98.1	104.2
6.5	121.7	94.1	101.0

are apparently straight lines. At pH 3.3 the conductivity increases consistently with the concentration of gelatin; at pH 5.1, however, the conductivity decreases strikingly with increasing amounts of gelatin. At pH 7.4 the conductivity again decreases with each increment of gelatin but the depression of the conductivity is less marked than at pH 5.1. Clearly the addition of gelatin at pH 3.3 to a solution of sodium chloride increases the conductivity of the solution; while the addition of gelatin near the isoelectric point (pH 5.1) decreases the conductivity in proportion to the amount of gelatin added. At the reaction of blood, also, the addition of gelatin to a sodium chloride solution depresses rather than increases the con-

[3] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

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ductivity of the resulting solutions. This latter result is evidence in favor of our original hypothesis that decreasing the percentage of protein in blood serum increases the conductivity of that serum.

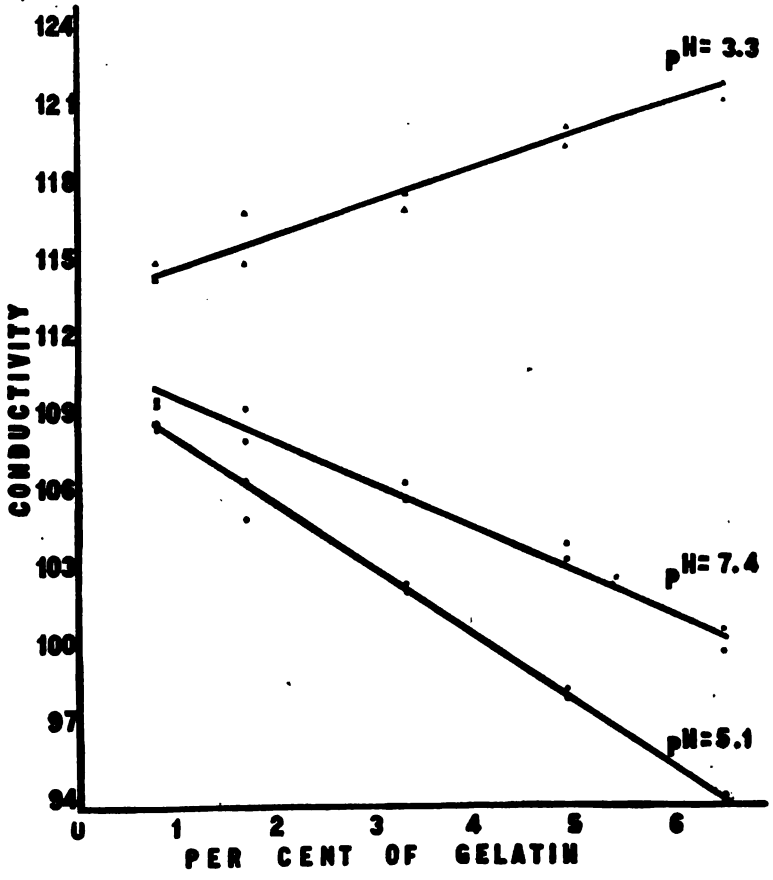

FIG. 2. Changes in conductivity of 0.6 per cent NaCl solutions with increasing concentration of gelatin (from 0.8 per cent to 6.5 per cent) at various hydrogen ion concentrations. At pH 3.3, the specific conductivity $\times 10^{-4}$ *increases* from 114.5 to 122. At pH 5.1, the conductivity *decreases* from 108.8 to 94.2. At pH 7.4 the conductivity *decreases* from 109.7 to 101.

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DISCUSSION.

The results charted in Fig. 2 may be explained as follows. The addition of gelatin to a solution of sodium chloride has two possible effects, which tend to oppose each other, (1) to increase the conductivity by the addition of ionized gelatin salts and (2) to decrease the conductivity by the mechanical interference of the large undissociated gelatin molecules. It could be predicted from the newer conceptions of the physical chemistry of the proteins that the first of these effects would be considerably greater at pH 3.3 than at pH 5.1, which is near the isoelectric point of gelatin and futhermore, it would be expected that the results obtained at pH 7.4 would be more nearly like the results observed at pH 5.1 than like those observed at pH 3.3. Whether the addition of gelatin at a given hydrogen ion concentration increases the conductivity or depresses it depends, therefore, upon the degree of ionization of the gelatin at that reaction. It is obvious that at the reaction of blood, the pure protein, gelatin, is so little ionized that the mechanical interference predominates and the conductivity decreases with each increment of gelatin.

CONCLUSIONS.

1. In pure gelatin solutions the conductivity of the solution increases with increasing concentrations, regardless of the hydrogen ion concentration. The actual value of the specific conductivity is greater at that reaction where the degree of ionization is greater.

2. The addition of gelatin in increasing concentrations to a 0.6 per cent sodium chloride solution affects the conductivity of that solution in two ways: (*a*) At pH 3.3, (where gelatin is highly ionized) the conductivity increases with each added increment of gelatin. (*b*) At pH 5.1 and 7.4 (where gelatin is less highly ionized) the conductivity decreases with each added increment of gelatin.

A similar study is being made of crystalline egg albumin.

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CORRELATION OF THE PROPAGATION-VELOCITY OF THE CONTRACTION-WAVE IN MUSCLE WITH THE ELECTRICAL CONDUCTIVITY OF THE SURROUNDING MEDIUM.

By SAMUEL ERNEST POND.

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(Received for publication, May 20, 1921.)

An attempt was made at Clark University in 1916 and 1917 to test experimentally the thesis that the rate of physiological conduction in irritable tissues is a direct function of the electrical conductivity of the medium in which the tissue acts. The investigation was a series of experiments with reference to the propagation-velocity of the contraction-wave in the heart muscle of the river terrapin under artificial conditions. Measurements of the velocity of the wave in a series of isotonic balanced salt solutions of graded activity (mixtures of Ringer's solution with isotonic sugar solution) were made by means of special apparatus. These preliminary results indicated a direct correlation between the propagation-velocity and the electrical conductivity, and that the ratio of velocity with respect to conductivity was nearly a constant until the concentration of the salts of the Ringer's solution was reduced more than half. The original apparatus has been modified and the investigation has been extended during 1919 and 1921 to include the heart of the king-crab, *Limulus*, and the sartorius of the leopard-frog, *Rana pipiens*. Observations have been made in the above solutions over an extended range of electrical conductivity and under nearly constant conditions of oxygen tension, hydrogen ion concentration, and temperature.[1]

Comparative observations upon the spreading of an excitation state in irritable tissues indicate that the speed of conduction is

[1] Pond, S. E., Contraction wave in heart-muscle of River Terrapin, Thesis Worcester, 1917.

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dependent principally upon the specific constitution of the tissue, the temperature, and the composition of the surrounding medium. What part the medium plays in the transmission of the contraction wave is not fully known.

Mayer[2] found that the rate of nerve conduction in the marine medusa *Cassiopea* was closely proportional to the total concentration of the cations Na, Ca, and K in the medium. When the sea water was diluted with distilled water a decline in the rate of nerve conduction was observed with increasing dilution. He attributed these results to the change in the adsorption of the above cations by the tissue; but it may also indicate, as Lillie pointed out[3], a direct correlation of the rate of physiological conduction with the electrical conductivity of the medium. In fact, the decline in propagation-rate runs closely parallel with the decline in the electrical conductivity of the sea water when similarly diluted. Mayer, however, found that under some conditions the propagation-rate may be independent of the electrical conductivity; thus a dilution of sea water with 0.4 *molar* magnesium chloride solution causes a decrease in the velocity of the nerve conduction to a degree closely proportional to the degree of dilution, although the electrical conductivity remains essentially unchanged.[4] The addition of magnesium however, must disturb the balance of the salts in the medium and introduce other factors of a special kind; and the possibility remains that in physiologically balanced media the rate of transmission of the excitation state may be determined, other conditions being equal, by the electrical conductivity of the solution.

In the light of these and other facts and of general theoretical considerations, Lillie advocates the theory "that the transmission of the excitation-state from the immediate site of activity to the adjoining resting areas is dependent on an *electrical local action* of the same essential nature as that which is responsible for the etching or corrosion of non-homogeneous metallic surfaces (*e.g.*, of iron) in con-

[2] Mayer, A. G., *Am. J. Physiol.*, 1915–16, xxxix, 375; 1916–17, xlii, 469; 1917 xliv, 591.

[3] Lillie, R. S., *Am. J. Physiol.*, 1916, xli, 133.

[4] Mayer, A. G., *Am. J. Physiol.*, 1915–16, xxxix, 381.

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tact with an electrolyte solution."[5] This hypothesis "assumes that the electromotor properties of the protoplasmic surface-film are determined by conditions which are fundamentally similar to those governing the electromotor phenomena at metallic surfaces."[6] With irritable tissues, just as with metallic elements under certain conditions, *e.g.*, passive iron in nitric acid solution, local alteration gives rise to a characterisitic spreading effect, which has a rate dependent partly upon the composition of the medium, and partly upon the peculiarities of the tissue or metal.[7] The effects of local stimulation in living tissues spreads to other parts at different rates, very rapidly in some tissues, *e.g.*, nerve, while in others, *e.g.*, smooth muscle, the rate of transmission is slow. Similarly there is a wide varation in the rate of electrolytic changes in metals under different conditions.[8, 9] The slow extension of a rust spot in iron in the presence of an electrolyte is an instance of a gradual spreading effect; while the change from the passive to the active state, in iron and other metals, spreads from a region of local alteration, under certain conditions, with great rapidity.[10, 11]

The general view that the bioelectric variation, as such, is the essential change on which conduction of excitation in irritable tissues depends is by no means a new one and was favored by du Bois-Reymond, Hermann, Kühne and other early students of the bioelectric phenomena.[12]

The present investigation relates to the rôle of the composition and electrical conductivity of the medium in the transmission of the contraction-wave in muscle.

[5] Lillie, R. S., *Am. J. Physiol.*, 1916, xli, 126.

[6] Lillie,[5] p. 129.

[7] Lillie, R. S. *Am. J. Physiol.*, 1914, xxxiv, 414; 1915, xxxvii, 348.

[8] Lillie, R. S., *Scient. Monthly*, 1919, viii, 456, 552. *Science*, N. S., 1919, l, 259, 416.

[9] Lillie, R. S., and Johnston, E. N., *Biol. Bull.* 1919, xxxvi, 225.

[10] Bennett, C. W. and Burnham, W. S., *J. Phys. Chem.* 1917, xxi, 107.

[11] Lillie, R. S., *Am. J. Physiol.*, 1915, xxxvii, 348; 1916, xli, 126; *J. Gen. Physiol.*, 1920, iii, 107.

[12] du Bois-Reymond, E., *Ges. Abhandl. allg. Muskel- und Nervenphysik*, 1888, ii, 698, 733; Kühne, W., *Proc. Roy. Soc. London*, 1888, xliv, 446; *Z. Biol.*, 1888, xxiv, 383.

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Method.

The velocity of the contraction-wave in muscle, *i.e.*, of the transmission of the excitation state, is measured by recording the time of the bulging of a strip of tissue at two or more points along its length as it undergoes contraction. In the present investigation the local bulging is made to operate (by a simple lever system) minute mirrors, which bring to focus upon a moving photographic film mirror-images of an illuminated slit. The muscle is either stimulated at one end by a minimal "break" induction-shock, or (as in the *Limulus* heart) it is rhythmically beating and the contraction-wave moving along the tissue operates the mirrors as it passes certain points. Each organ or strip of muscle while fixed in place for experimentation is arranged so that without otherwise changing the conditions it may be exposed to a succession of solutions of graded electrical conductivity; *e.g.*, mixtures of sea water or Ringer's solution with isotonic sugar solution in varying proportions (1:9, 2:8, 3:7, *etc.*). An electrically actuated tuning-fork of suitable frequency, with an attached mirror, is so arranged that a time curve is recorded upon the moving film above the muscle record. The solutions used with turtle and frog tissues have been corrected to, or kept at, constant temperature, about 20°C., and the hydrogen ion concentration of the solutions has been adjusted before experimentation to that of the Ringer's solution employed in the same series; *i.e.*, about pH = 6.8. Measurements of the hydrogen ion concentrations have been made by the colorimetric method of Clark and Lubs with standardized buffer solutions.[13] The electrical conductivity of the solutions in all the work has been measured at 25°C. by the method of Kohlrausch, determinations being made immediately before and after taking the muscle record.

The apparatus which has been used in this study for recording the passage of the contraction-wave is specially constructed so as to allow the external medium to be changed at will without otherwise disturbing the tissue. It is described in detail by the author elsewhere. The supporting part of the apparatus coming into direct contact with the frog and turtle tissues is entirely composed of vulcanized

[13] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 38ff.

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fiber impregnated with Bakelite; this substance is insoluble in the solutions used, and does not affect the tissues. In the experiments with the heart of *Limulus* the organ was fixed in a trough composed of paraffin.

The tissues were prepared as follows: Frogs were first curarized, with about 2 cc. of a 1 per cent solution of curare (in Ringer's solution), and after a period of 20 minutes both sartorii were removed. One of the muscles was mounted on the support and the other kept in cold Ringer's solution. The companion muscle was used in a few cases in duplicate experiments. The ventricle of the turtle was removed, cut into rings, and two of these opened up as strips of thick muscular tissue. One was placed in position on the apparatus and the other kept reserve for later use. The heart of the female *Limulus* (in preference to the male because of its greater length) was removed through a dorsal opening in the carapace and placed in the paraffin trough with the ventral side up, so that the ganglion was completely immersed in the solution. On one occasion, the muscle tissue was cut away from the ganglion between the third and sixth segments, leaving the anterior and posterior segments connected by the ganglion.

Complete records of one or more muscle contraction waves were taken upon a strip of photographic film wrapped about a kymograph drum. The procedure adopted was as follows: The tissue was placed in position for a record, washed in at least two changes of the test solution over a period of 15 or 20 minutes, and then exposed to a final bath in which the records were taken. *Limulus* hearts in the troughs were so arranged that the test solution flowed slowly over and about the tissue; while the turtle and frog tissues were exposed in tumblers holding about 250 cc. of solution. The arc-lamp was lighted and adjusted to uniform illumination of the mirrors; the tuning-fork was then started and the kymograph brought up to speed. The latter was so arranged (inside of a light-proof box) that a shutter on the arc-lamp remained open during one complete revolution of the drum. An inductorium circuit was closed (in response to a signal attached to the kymograph) shortly before the shutter was opened and broken just afterward. Thus during one revolution of the kymograph drum the tissue was stimulated and one complete muscle record was made, together with the time curve.

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Thre curves, those of the tuning-fork and of the contraction-waves at two points of the muscle were thus described above one another. Experiments with the rhythmically beating heart of *Limulus* required opening the shutters by hand just before a contraction of the organ.

Determinations were made to ascertain the time which should elapse after a change of solution in order to enable the tissue to reach a uniform condition and permit duplicate records to be secured. This period, with frequent changes and stirring of the solutions in the tumblers, was rarely less than 20 minutes in the smallest frog muscle. During this time the records indicated gradual changes in the speed of the contraction-wave. After a lapse of this initial period records were taken at intervals duringan hour or more. These related to the behavior of the tissue (*a*) in the normal medium, Ringer's solution or sea water, (*b*) in a medium made by diluting the normal medium with isotonic sugar solution, and (*c*) again in the nor mal medium. The change in the behavior of the tissue on passing from the normal medium of high conductivity to a medium of lower conductivity could thus be compared with the change following the reverse transfer. The rate of recovery of propagation-velocity on returning from the medium of low conductivity to the normal medium could also be determined.

RESULTS.

Limulus.—The experiments upon the heart of the king-crab were designed to show (*a*) changes of irritability in pure isotonic sugar solutions, (*b*) the behavior in mixtures of sea water with isotonic sugar solution in various proportions, and (*c*) the behavior in sea water under the same experimental conditions over a period equivalent to that covered by the tests. The determinations of the propagation-velocity have been made over different distances and with respect to different portions of the hearts. The most consistent records are from the anterior third; while those from the posterior and anterior region, *i.e.*, covering the length of the whole organ, are of doubtful value. In all cases the temperature of the solutions employed has been adjusted to that of the sea water on the day of the experiment.

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In running sea water the rate of beat of the excised heart may differ from that observed in the body before the operation. After transfer to the paraffin trough used in the tests the rate of beat at first decreases, or the heart may stop beating altogether. In the latter case hearts usually resume beating in a few minutes after slight pinching or tapping. The rate of beat was always found to increase during the next 10 minutes, and, after this interval it usually remained essentially unchanged. In some cases a decrease in the rate of beat was observed, and the amplitude of the contraction decreased, although the excised heart will frequently beat for 2 days or more. If now, after 30 minutes in sea water, the heart is placed in pure isotonic sugar solution, the beat at first slightly increases in rate and then diminishes through a period of 70 to 80 minutes and finally ceases altogether. In six hearts observed under these conditions the average time required for a complete loss of rhythmic beating was 76 minutes. Artificial stimulation, pinching or turning the heart about in the trough did not induce any regular beating. Rhythmical beating was resumed, however, as soon as sea water was allowed to flow about the heart for a few moments; the rate of beat was in some instances somewhat slow at first but soon became about the same as before exposure to the sugar solution. Two of the above hearts which had been exposed to sugar solution for a little over 2 hours recovered in sea water within 3 minutes; one heart exposed to sugar solution for $3\frac{1}{2}$ hours; recovered in a little less that 4 minutes. The three remaining hearts were returned to sea water within a few minutes after the rhythmic beating had stopped and recovered in between $2\frac{3}{4}$ and 3 minutes. Duplicate records of the speed of the contraction-wave were not secured in these experiments because of the variability of the beating in sugar. In solutions in which three-quarters of the sea water is replaced by sugar solution, *i.e.*, 25 per cent sea water or less, there is a constant decrement in the rhythmic beating and a final loss of irritability; the time required for this loss is greater than in pure sugar solution, varying from 2 to $3\frac{1}{2}$ hours. Attempts to get duplicate records of the propagation-velocity in these solutions were not successful. Some observations were made in 30 per cent sea water, but the velocity of the contraction-wave over two or three segments proved exceedingly slow and the observations showed poor agreement.

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Most determinations of the propagation-velocities have accordingly been made in mixtures of sea water and sugar solution containing 40 per cent or more of the normal electrolyte content; *i.e.*, 0.4 or more sea water.

Estimates of the velocity of propagation of the contraction-wave in the *Limulus* heart based upon records taken from the two opposite ends of the organ are variable and inconsistent. Records taken from the second and eighth segments also appear in no cases to have given consistent results. If recording devices are attached to the second,

TABLE I.

Contraction-Wave in Limulus Heart. Experiment 30. Sea Water, 21°C.

Distance.	Velocity per second.	Segments.	Time.
cm.	*cm.*		*hrs.*
3.0	75	4→2	1
4.4	62	4→8	1
7.4		8→2	1
3.0	69	4→2	3
4.4	65	4→8	3
7.4		8→2	3
3.0	75	4→2	6
4.4	65	4→8	6
7.4		2→8	6
3.0	68	4→2	8
4.4	64	4→8	8
7.4		2→8	8
3.0	75	4→2	10
4.4	65	4→8	10
7.4		2→8	10

fourth and eighth segments as recorded in Table I, the records show that at times the contraction of the second segment may actually precede that of the eighth, while the intermediate segment may contract before either of the others. Hence the time elapsing between the contractions at opposite ends of the heart may be so brief as to show that the case is not one of simple transmission of a single contraction-wave from end to end of the organ. Transmission, however appears to be uniform over a short region near the middle of the heart. Records from this middle region, *i.e.*, fourth, fifth, and sixth segments, indicate that these segments contract almost simultaneously.

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The majority of the records have been taken from two points situated a short distance apart in the middle region. The results are not combined in a single set of figures because the same conditions do not appear to prevail in all regions. Each experiment by itself however, indicates that a lowered electrical conductivity of the medium is always associated with a reduction in the propagation-velocity of the contraction-wave over the region under observation. It will be noted that in all of the experiments the ratios of velocity with respect to conductivity are of the same order.

Another variable feature has been encountered in the irregular behavior of the hearts of animals which were kept in captivity for more than two months at Woods Hole. After being exposed in the trough for a few hours such hearts would often reverse their direction of beat, or would become arhythmical. In the case of animals fresh from the shore and others which had been artificially fed with small fish the hearts showed regular behavior and have provided consistent records, from which Table II has been compiled.

In running sea water the propagation-velocity of a healthy heart may be regularly so high as 80 cm. per second, but this velocity is not always found if the same regions are compared. In the eight experiments reported the velocity varies from 63 to 81 cm. per second. The average velocity is in the neighborhood of 70 cm. per second. The concentrations given in the second column of Table II are expressed as the fractional part of sea water present in the mixed solutions, *i.e.*, a mixture of 8 parts sea water and 2 parts isotonic sugar solution is designated as "0.8 *Sw.*" The conductivity of the sea water is taken as unity, and the proportional conductivity of the mixtures if calculated from the experimental determinations of resistance. The velocity of the propagated disturbance is expressed in centimeters per second. These two sets of determinations are combined in the last column as the ratio of velocity v with respect to conductivity c. All of the calculations are subject to an error introduced in reading the records, varying from 5 per cent at the speed of 80 cm. per second to 2 per cent at 20 cm. per second.

A number of observations were made with reference to the aeration of the solution. *Limulus* hearts examined in mixed solutions made with sea water which had been shipped to Worcester from

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TABLE II.

Contraction-Wave in Limulus Heart.

Record.	Experiment.	Solution.	Conductivity. (Sw 1).	Temperature.	Distance.	Velocity per second.	$\frac{v}{c} = k.$	Segments.
				°C.	cm.	cm.		
C	32	*Sw*	1.00	22.0	4.0	81	81	5→2
D	32	0.7*Sw*	0.66	22.0	4.0	55	83	5→2
E	32	*Sw*	1.00	22.0	4.0	83	83	5→2
H	32	0.5*Sw*	0.55	22.0	4.0	43	78	5→2
K	32	*Sw*	1.00	22.0	4.0	76	76	5→2
B	35	*Sw*	1.00	22.0	3.5	78	78	5→2
F	35	0.8*Sw*	0.77	22.0	3.5	57	74	5→2
J	35	*Sw*	1.00	22.0	3.5	73	73	5→2
B	36	*Sw*	1.00	22.0	5.0	78	78	6→2
F	36	0.5*Sw*	0.55	22.0	5.0	42	61	6→2
H	36	*Sw*	1.00	22.0	5.0	74	74	6→2
C	44	*Sw*	1.00	22.0	3.5	73	73	5→2
E	44	0.7*Sw*	0.66	22.0	3.5	42	64	5→2
G	44	*Sw*	1.00	22.0	3.5	73	73	5→2
I	44	0.7*Sw*	0.66	22.0	3.5	42	64	5→2
K	44	*Sw*	1.00	22.0	3.5	67	67	5→2
B	52	*Sw*	1.00	21.5	3.1	70	70	4→2
D	52	0.5*Sw*	0.55	21.5	3.1	35	64	4→2
F	52	*Sw*	1.00	21.5	3.1	60	60	4→2
I	52	0.7*Sw*	0.66	21.5	3.1	49	73	4→2
L	52	*Sw*	1.00	21.5	3.1	60	60	4→2
B	53	*Sw*	1.00	21.5	4.3	63	63	4→8
D	53	0.9*Sw*	0.92	21.5	4.3	60	65	4→8
F	53	*Sw*	1.00	21.5	4.3	63	63	4→8
I	53	0.7*Sw*	0.66	21.5	4.3	46	69	4→8
K	53	*Sw*	1.00	21.5	4.3	63	63	4→8
N	53	0.5*Sw*	0.55	21.5	4.3	36	65	4→8
P	53	*Sw*	1.00	21.5	4.3	60	60	4→8
B	54	*Sw*	1.00	21.5	4.0	63	63	5→2
D	54	0.8*Sw*	0.77	21.5	4.0	55	72	5→2
G	54	*Sw*	1.00	21.5	4.0	63	63	5→2
J	54	0.4*Sw*	0.42	21.5	4.0	24	58	5→2
L	54	*Sw*	1.00	21.5	4.0	63	63	5→2
O	54	0.6*Sw*	0.62	21.5	4.0	38	62	5→2
R	54	*Sw*	1.00	21.5	4.0	59	59	5→2
C	55	*Sw*	1.00	21.5	3.0	68	68	4→2
E	55	0.7*Sw*	0.66	21.5	3.0	47	71	4→2
G	55	*Sw*	1.00	21.5	3.0	68	68	4→2
I	55	0.4*Sw*	0.42	21.5	3.0	29	69	4→2
K	55	*Sw*	1.00	21.5	3.0	63	63	4→2
M	55	0.6*Sw*	0.62	21.5	3.0	44	71	4→2
P	55	*Sw*	1.00	21.5	3.0	63	63	4→2

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Woods Hole, exhibited a smaller decrement in the rate of beat when air had been bubbled through the solutions than when this procedure was omitted. At Woods Hole the records showed better agreement when the behavior of the hearts was studied in freshly made mixtures or in aerated mixtures than when similar solutions were used which had stood in bottles for 2 days or more.

Frog.—More extensive studies of the relationship between the electrical conductivity of the surrounding medium and the propagation-velocity of the contraction-wave have been made with the sartorius muscle of the leopard-frog. Each muscle strip is mounted vertically on a supporting apparatus; the muscle is stimulated electrically at one end by minimal single shocks, and the contraction-wave travels thence to the other end of the tissue along the parallel fibers. The distances between the two points at which the wave has been recorded vary between 1.5 cm. and 2.4 cm. Break-shocks are sent into the tendinous end of the muscle through a platinum stimulating electrode. The temperature during the experiments has been adjusted to 20°C. In some cases reported below the oxygen tension has been increased by bubbling oxygen through the solutions. The hydrogen ion concentration of all the mixtures is nearly 6.8.

Tests made over long periods of time indicate that the sartorius muscle of the frog is very resistant to the artificial conditions imposed in the experiments. Observations on muscles immersed in Ringer's solution have been made at intervals during periods so long as 96 hours, and toward the end of this time only a slight loss in the propagation-velocity of the contraction-wave has been recorded. The amplitude of the contraction-wave appears to decrease very gradually during the first 2 days, and more rapidly during the third and fourth days. In solutions of cane-sugar the irritability of the tissue is soon lost completely; the time required with several changes of solution varies from 20 to 30 minutes. Upon return to Ringer's solution the irritability is restored within approximately 1 minute. At 20°C. the normal rate of conduction of the tissue in Ringer's solution is about 3 m. per second. In the Ringer-sugar mixtures this rate decreases in close proportionality with the reduction of the electrical conductivity until the concentration of the salts has been reduced to about one-half the normal. In the range between the

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dilution with one-half reduction of salts to three-fourths reduction, the propagation-rate falls off more rapidly than the electrical conductivity; while if more of the Ringer salts be replaced by sugar the irritability is soon decreased to zero. In the solutions of low electrical conductivity the tissue becomes non-irritable in the course of 2 hours or more, but recovers immediately upon being replaced in Ringer's solution. It is remarkable that increase of oxygen tension may largely compensate for the reduction in salts. If oxygen be bubbled through the solutions the decrease of propagation-velocity may remain almost proportional to the decrease of electrical conductivity until only one-twentieth of the salts of Ringer's solution remain (*cf.* Table V).

Seventy-four series of experiments have been conducted upon propagation-velocities in varying mixtures of Ringer's solution and isotonic sugar solution. The order of the solutions used has been frequently altered to offset errors; while the time between the transfer of the muscle to a given solution and the taking of the record has always been long enough to allow the tissue to reach an equilibrium with the medium. Experiments 141, 142, and 143 (*cf.* Table III) are typical of the observations, and are selected with reference to the mixtures ranging from 30 per cent Ringer's solution to normal Ringer. The velocities and the conductivities are averaged and the ratios calculated in Table IV. In these experiments the mixtures were agitated continuously with a slow stream of air.

Experiments have been made on the velocity of the contraction-wave in solutions of still lower electrical conductivity, in which an increase of oxygen tension enables transmission to occur. Nine experiments in which oxygen was bubbled through the mixtures are recorded in Tables V and VI. In three of these experiments a mixture was employed containing only 5 per cent of the normal salt content of Ringer's solution. The muscle contracted and transmitted the contraction wave under these conditions; although in the same solutions containing oxygen at air tension no such behavior appears possible. In pure M/4 sugar solution saturated with oxygen these muscles lost irritability in slightly less than a half hour. Two of the experiments in the 0.05 Ringer's solution show a transmission rate very close to that calculated from the electrical conductivity.

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TABLE III.

Contraction-Wave in Sartorius of Frog.

Record.	Solution.	Conductivity.	Velocity.	$\frac{v}{c} = k$.
Experiment 141; Wave Distance = 1.6 cm.; 20° C.				
B	Ringer	1.00	319	319
D	0.6R	0.58	174	300
G	0.4R	0.38	104	276
J	Ringer	1.00	319	319
Experiment 142; Wave Distance = 1.6 cm.; 20° C.				
A	Ringer	1.00	320	320
C	0.8R	0.81	258	319
D	0.5R	0.48	140	291
G	Ringer	1.00	320	320
J	0.3R	0.30	79	262
L	0.7R	0.69	215	312
N	Ringer	1.00	320	320
P	0.9R	0.92	288	313
R	Ringer	1.00	320	320
Experiment 143; Wave Distance = 1.7 cm.; 0 20° C.				
A	Ringer	1.00	306	306
G	0.3R	0.30	90	300
J	Ringer	1.00	306	306
M	0.9R	0.92	278	302
R	0.5R	0.48	127	265
S	Ringer	1.00	306	306
X	0.7R	0.69	213	309
Z	Ringer	1.00	306	306

TABLE IV.

Contraction-Wave in Sartorius of Frog.

(Average of Experiments 141, 142, 143.)

Solution.	Conductivity.	Velocity.	$\frac{v}{c} = k$.
Ringer	1.00	315	315
0.9R	0.92	283	308
0.8R	0.81	258	318
0.7R	0.69	214	310
0.6R	0.58	174	300
0.5R	0.48	134	279
0.4R	0.38	104	274
0.3R	0.30	85	283

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In all of the solutions having a conductivity less than seven-tenths that of Ringer's solution the average ratio of velocity with respect to conductivity is higher than in Ringer's solution (*cf.* Table VI).

TABLE V.

Contraction-Wave in Sartorius of Frog.

(With Constant Stream of Oxygen.)

Solution.	Conductivity. (Ringer = 1.0)	Velocity per second.								
		Experiment No.								
		94	95	96	97	98	99	101	103	107
		cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
Ringer	1.00	295	310	315	312	300	320	300	306	315
0.9R	0.92	—	—	—	—	—	—	—	271	280
0.8R	0.81	—	246	—	250	—	—	245	—	—
0.7R	0.69	—	—	—	—	—	—	—	220	214
0.5R	0.48	150	—	160	—	144	—	147	—	155
0.4R	0.38	—	125	—	122	—	130	—	119	126
0.3R	0.30	100	—	—	—	—	—	95	—	—
0.2R	0.20	—	70	73	—	110	60	—	75	70
0.1R	0.11	40	—	45	—	—	35	—	—	—
0.05R	0.06	—	20	22	37	—	—	—	—	—
0.00R	0.0001	0	0	0	0	—	—	—	—	—

TABLE VI.

Contraction-Wave in Sartorius of Frog.

(Average Velocities of Table V.)

Solution.	Conductivity. (Ringer = 1.0)	Velocity per second.	$\frac{v}{c} = k.$
		cm.	
Ringer	1.00	308	308
0.9R	0.92	276	300
0.8R	0.81	247	304
0.7R	0.69	217	315
0.5R	0.48	151	314
0.4R	0.38	124	326
0.3R	0.30	97	323
0.2R	0.20	76	380
0.1R	0.11	40	458
0.05R	0.06	26	433

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TABLE VII.

Contraction-Wave in Ventricle of Turtle.

Record.	Solution.	Conductivity (Ringer = 1.00).	Velocity per second.	$\frac{v}{\epsilon} = k$.
			cm.	
Experiment 152; Wave Distance = 2.0 cm.; 20°C.				
A	Ringer	1.00	7.6	7.6
D	0.5R	0.48	5.9	12.3
F	0.7R	0.69	6.4	9.2
H	Ringer	1.00	7.8	7.8
Experiment 154; Wave Distance = 1.6 cm.; 20°C.				
B	Ringer	1.00	8.8	8.8
E	0.8R	0.81	6.7	8.3
H	0.5R	0.48	4.0	8.3
L	0.4R	0.38	2.8	7.4
N	0.6R	0.58	4.8	8.3
P	Ringer	1.00	7.2	7.2
Experiment 155; Wave Distance = 3.0 cm.; 20°C.				
C	Ringer	1.00	9.1	9.1
E	0.4R	0.38	3.4	8.9
F	Ringer	1.00	7.5	7.5
Experiment 156; Wave Distance = 3.0 cm.; 20°C.				
C	Ringer	1.00	9.9	9.9
E	0.7R	0.69	6.0	8.7
H	0.9R	0.92	6.8	9.8
J	Ringer	1.00	8.8	8.8
M	0.5R	0.48	4.6	9.6
R	Ringer	1.00	6.5	6.5
Experiment 158; Wave Distance = 2.4 cm.; 20°C.				
B	Ringer	1.00	7.4	7.4
F	0.8R	0.81	7.1	8.7
G	0.6R	0.58	5.2	8.9
I	Ringer	1.00	6.9	6.9

Turtle.—Strips cut from the ventricle of the river terrapin were used in 1917 and 1921 with two different recording devices. The earlier observations were recorded by means of levers writing on smoked paper; in the later experiments the optical recording device

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described above was used. In both sets of observations the initial speed is practically the same at about 20°C.; *viz.* about 7 cm. per second. Both the old and the newer readings agree also in respect to the rate and character of the changes undergone by the tissue under the experimental conditions. After a period of 6 hours in Ringer's solution the transmission-velocity begins to be irregular and the minimal stimulus in Ringer may fail to produce contraction. Upon changing the tissue end for end, or cutting off a little of the tissue on both ends, the regularity of response to the original minimal stimulus is restored and the transmission-velocity recovers. The strips of tissue selected for use have been from 3 to 5 mm. wide and from 3.5 to 4.5 cm. long. The observation points were between 0.6 and 3 cm. apart.

In 0.3 Ringer's solution strips of ventricle lose their irritability in the course of about 2 hours, and in pure isotonic sugar solution in about 50 minutes. Recovery of irritability upon replacement in normal Ringer's solution occurs in about 2 minutes. Little difference has been observed between the propagation-velocities in freshly prepared Ringer's solution and in older solutions through which air was bubbled.

The velocity of the contraction-wave is reduced in mixtures of Ringer with sugar solution; but in solutions of less than 0.6 Ringer the reduction of transmission-velocity is not proportional to the reduction in conductivity. Five experiments of the last series are recorded in Table VII.

DISCUSSION.

The data presented in this paper lend further support to the thesis that the electrical conductivity of the medium surrounding an activated protoplasmic system is in direct correlation with the rate of spread of the state of activation over the system. In skeletal muscle, the contraction-wave travels along the fibers at a velocity which is closely proportional to the electrical conductivity of the fluid in contact with it. A reduction, by means of dilution with an indifferent non-electrolyte solution, in the electrical conductivity of the normal balanced medium is followed by a corresponding reduction in the propagation-velocity of the contraction-wave. This parallelism

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is closer in solutions of higher than in those of lower electrical conductivity. The ratio of velocity with respect to conductivity is nearly a constant with frog's muscle, and less constant with muscles of the turtle and *Limulus*.

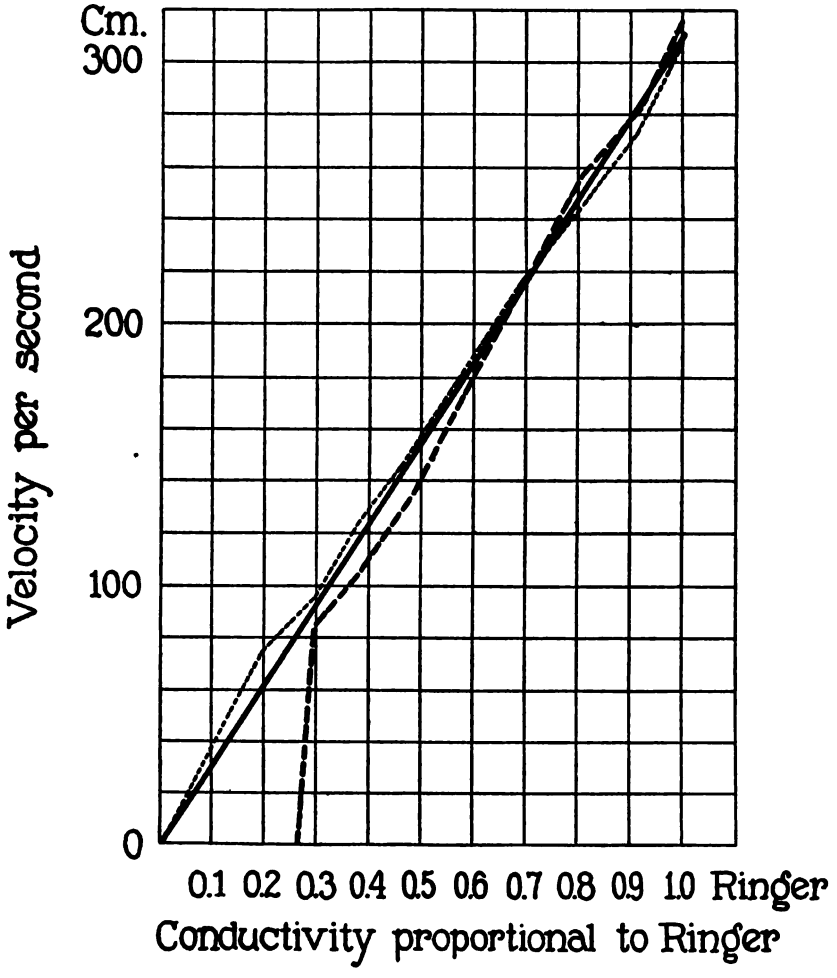

FIG. 1. Sartorius of frog in Ringer-sugar solution. Long dashes indicate aerated mixtures (Table IV); short dashes, oxygenated mixtures (Table VI).

In Fig. 1 the velocities of the contraction-wave in the sartorius of the frog are plotted against the conductivities of the solutions.

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It will be seen that the dotted line runs closely parallel with the straight line which represents an ideal dependence of speed of conduction upon electrical conductivity. The dotted line connects the values secured when the muscle is immersed in a solution saturated with oxygen; while the dash line connects those obtained when the muscle is exposed to media containing oxygen at air tension.

The fact that in the presence of a stream of oxygen the transmission of the contraction-wave is possible even in solutions of very low conductivity is an indication that processes of oxidation are directly concerned in stimulation and in the propagation of the excitation state. If the primary change in stimulation is an alteration of the protoplasmic surface film, it would appear that in a medium of a given electrical conductivity a certain minimal concentration of oxygen is necessary for the completion of this surface reaction. Lillie has pointed out that such a relation of oxygen to a chemical reaction at the cell surface is suggestive of conditions similar to those of an electrolysis at an electrode. In this case it is "possible to reduce the current-strength through a wider range, and still have a high rate of decomposition at the electrode, if the concentration of the reacting substance is high, than if it is low."[14] In the present state of knowledge of the chemical conditions determining the formation of an active or "stimulated" region in living tissue, it is difficult to define clearly the rôle of oxygen in the stimulation process. In general it seems most probable that free oxygen is required in the return of the stimulated region to the resting state and in the propagation of the contraction-wave after the local stimulation has been aroused. If the analogy between activation in living tissues and in oxidisable metals bathed by electrolyte solutions, *e.g.* iron in nitric acid, is at all close, then the destruction of the surface film at any region during the rise of the activation-wave should depend upon a local reduction rather than upon an oxidation; apparently when the reduction has reached its maximum an oxidation process occurs, reforming the film and the passive or resting state is regained. In other words, the local excitation state is to be regarded as an effect resulting from the expenditure of energy in the stimulated region—

[14] Personal communication.

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accompanying a reduction process; after the excited region has reached a maximum negativity (electrically negative with respect to adjacent regions) the reverse or oxidative process automatically follows, with the effect of reestablishing the passive or resting state. According to this hypothesis the free oxygen enters directly into only one part of the local stimulating process; but this oxidation must be repeated at each successive region during the propagation of the excitation state along the tissue.

If transmission in the living tissue is in fact dependent upon secondary electric stimulation of the resting region by the local bioelectric current between that region and the active region adjoining, there should be a direct proportionality between the electrical conductivity of the medium and the rate at which the state of activity spreads from region to region. The electrical conductivity of the first local stimulating circuit, other conditions being equal, determines the intensity of the current at any point in the circuit; and if the conductivity is uniform throughout the tissue and medium the rate of propagation will be uniform throughout the tissue. Differences in the electrical conductivity of the medium will be associated with differences in the rate of propagation because of the effect upon the intensity of the local bioelectric current traversing the tissue at any point adjacent to the active (electrically negative) area. The greater the conductivity of the medium, the greater will be the distance (from the already active region) at which the current traversing the resting tissue is sufficiently intense to effect the stimulating reaction. It is on the basis of such general considerations that Lillie believes we may provisionally disregard the special chemical nature of the local reaction (at the cell-medium boundary), on the ground that at present we know very little about it. Whatever the nature of the local stimulation process may happen to be, if it is initiated electrically by the current of the local bioelectric circuit it should spread from region to region at a rate proportional to the electrical conductivity of the circuit. This conductivity depends chiefly on the conductivity of the external medium.

In the preliminary work before any oxygen had been employed experimentally and when conditions were constant (as with air bubbled through the solutions or with fresh solutions) it seemed

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possible that sugar might have some direct toxic action upon the tissues. Later considerations, however, make this doubtful. In the first place the parallelism between the electrical conductivity and propagation rate is closer with higher than with lower tensions of oxygen, although the sugar solutions are of the same concentration. In the second place if sugar acts toxically the toxic effect should increase proportionately to the increase in the length of exposure to the more concentrated sugar solutions. That this is not the case is indicated by the fact that the frog and *Limulus* tissues after immersion in pure sugar solution for periods up to 3 and 4 hours recover their irritability, upon return to Ringer's solution, just as rapidly and completely as when exposed to the pure sugar solution for periods just sufficient to abolish irritability. A further peculiarity in the action of sugar solutions, namely, the marked difference between the time required for loss of irritability in sugar solutions and for recovery of irritability on return to the normal conducting solution—the latter interval being many times shorter than the former—is not readily explained. It is beyond the aim of the present paper to discuss this problem, and it may be that the conditions must be more carefully investigated.

The author desires to thank Dr. Ralph S. Lillie for constant help and advice in the study of the present problem, and the Trustees of Clark University for the arrangements under which the research has been prosecuted.

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DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

III. Viscosity.

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I. Volume of Solute and Viscosity.

The viscosity of freshly prepared gelatin solutions is affected in a similar way by the pH as are the electromotive forces, the osmotic pressure, and the swelling. We have been able to show that the influence of the pH on the E.M.F. can be accounted for quantitatively on the basis of the Donnan equilibrium,[1] and that with the exception of one or two minor deviations the same is true for the osmotic pressure.[2] Procter and Wilson's theory of swelling is also based on Donnan's theory of membrane equilibrium.[3] Fig. 1 is the expression of the influence of the pH on the viscosity of 0.5, 1, and 2 per cent freshly prepared gelatin chloride solutions at a temperature of 24°C. The abscissæ are the pH of the gelatin solution, while the ordinates are the relative viscosities of the gelatin solutions compared with that of water at the temperature of the experiment.

These curves are modified if the viscosity of the gelatin solution is not measured immediately, but only after the solution has been standing for some time. In this case the curve changes inasmuch as the viscosity rises everywhere but the more rapidly, the nearer the pH is to that of the isoelectric point. In this case the curves representing the influence of pH on the viscosity of gelatin solution no longer resemble the curves representing the influence of the pH

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 577, 667.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 691.

[3] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

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on the osmotic pressure and swelling. In the literature, however, it is usually stated that the influence of acid on viscosity resembles that of acids on osmotic pressure and swelling, and the question arises whether or not the theory of the Donnan equilibrium can be applied to the explanation of this type of viscosity curves found when freshly prepared gelatin solutions are used (Fig. 1).

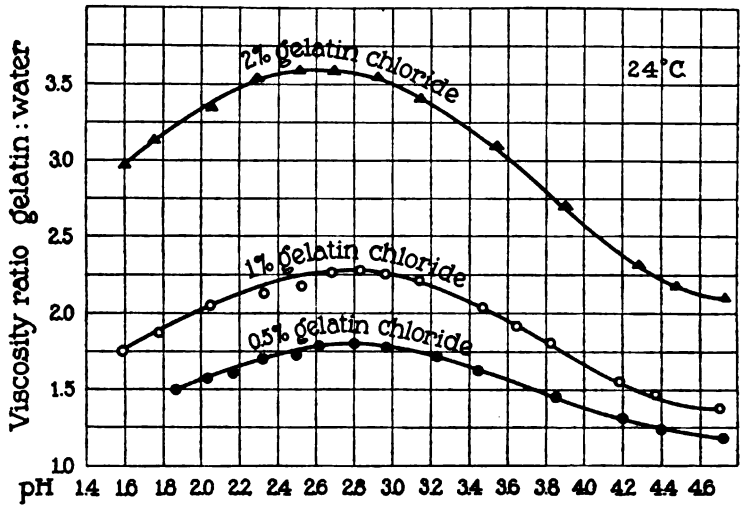

FIG. 1. Influence of pH on viscosity of freshly prepared gelatin chloride solutions.

Several formulæ exist for the calculation of the influence of a solute on the viscosity of a solvent. The first one (1) was derived by Einstein[4]

$$\eta = \eta_0 (1 + 2.5 \varphi) \qquad (1)$$

where η_0 is the viscosity of the pure solvent at the temperature of the experiment, η the viscosity of the solution, and φ the fraction of the volume occupied by the solute in the solution. This formula holds only when φ is very small and when the particles of the solute are spherical and large compared with the molecules of the solvent.

[4] Einstein, A., *Ann. Physik*, 1906, xix, 289; 1911, xxxiv, 591.

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Hatschek,[5] Smoluchowski,[6] Hess,[7] and Arrhenius[8] have modified Einstein's formula so as to make it valid for any concentration. Arrhenius replaces the linear by a logarithmic formula

$$\text{Log } \eta - \text{Log } \eta_0 = \theta\varphi \qquad (2)$$

where φ is again the fraction of volume occupied by the solute in the solution and θ a constant, while η and η_0 have the same significance as in Einstein's formula.

All the formulæ agree in one point, namely that the fraction of the volume occupied by the solute in the solution is the main variable upon which the relative viscosity of a solution depends. It has been pointed out by Odén[9] and others that in addition to the relative volume occupied by suspended particles the average size of the individual granules in a suspension plays also a rôle in viscosity. According to these theories of viscosity, it should be possible to correlate the characteristic influence of the hydrogen ion concentration upon the viscosity of gelatin solutions with a variation in the relative volume or the average size of the gelatin particles in solution, since the mass of gelatin in solution remains the same in these experiments.

The measurement of the viscosity is in our experiments the time of outflow of the solution through a capillary tube and the method of the experiments (already described in a previous paper) was briefly as follows. To 50 cc. of a 2 per cent solution of isoelectric gelatin is added the desired acid, *e.g.*, HCl, in sufficient quantity and then the volume is raised to 100 cc. by the addition of enough distilled water. This 1 per cent solution of originally isoelectric gelatin is rapidly heated to 45°C., kept at that temperature for 1 minute, and then rapidly cooled to 24°C. (or any other desired temperature). The viscosity is measured immediately after the solution was cooled to 24°C., since on standing the viscosity increases unequally at different pH. The measurements were all made by determining the time of outflow through a capillary tube. The time of

[5] Hatschek, E., *Kolloid Z.*, 1913, xii, 238; 1920, xxvii, 163.

[6] Smoluchowski, M. v., *Kolloid Z.*, 1916, xviii, 190.

[7] Hess, W. R., *Kolloid Z.*, 1920, xxvii, 1, 154.

[8] Arrhenius, S., *Meddelanden from K. Vetenskapsakademiens Nobelinstitut*, 1917, iii, No. 21.

[9] Odén, S., *Nova acta regiae Societatis Scientiarum Upsaliensis*, 1913, iii, No. 4.

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outflow for pure water was 56 seconds at 24°C. The pH of the solutions was determined with the aid of the potentiometer.

The reader will notice (Fig. 1) that the relative viscosity of a gelatin solution is a minimum at the isoelectric point (pH=4.7), that it rises with a rise in the hydrogen ion concentration until it becomes a maximum at pH of about 2.7, and that it drops again with a further rise in the hydrogen ion concentration. The question is how to explain the apparent changes in the relative volume of the gelatin in solution which, according to the theory, must be the main cause of the variation of the viscosity with the pH.

A change in the ratio of volume of gelatin to volume of water is only possible if water is added to the gelatin. Pauli[10] suggested that the ionized particle of protein is surrounded by a shell of water which is lacking in the non-ionized molecule. The volume of the protein ions in solution is increased by this jacket of water. Since the gelatin in solution is practically non-ionized at the isoelectric point, the relative volume of the gelatin in solution is a minimum at this point, while when we add an acid, *e.g.*, HCl, gelatin chloride is formed, which, like all salts, ionizes readily. On this basis we can understand why the viscosity should increase with an increase of the hydrogen ion concentration of the gelatin solution; since with this increase in the hydrogen ion concentration, the concentration of hydrated gelatin ions and hence the volume of the gelatin particles should also increase. The work of Lorenz,[11] Born,[12] and others casts, however, a doubt on the assumption of a general hydration of polyatomic ions. We shall see presently that there are still other facts which show that the mere ionization and consequent hydration of the individual protein ions cannot well be the cause of the influence of the pH on the relative viscosity of gelatin solutions.

II. The Influence of the Hydrogen Ion Concentration on the Viscosity of Solutions of Amino-Acids and of Crystalline Egg Albumin.

If hydration of the individual protein ions were the cause of the variation of the viscosity of gelatin solutions, a variation of the hy-

[10] Pauli, W., *Kolloid Z.*, 1913, xii, 222.
[11] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.
[12] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

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drogen ion concentration should have a similar influence on the viscosity of solutions of simple amino-acids, like glycocoll and alanine, as it has on the viscosity of gelatin solution. 5 per cent solutions of glycocoll and alanine were brought to different pH, from 5.0 to 2.0 and below, by the addition of HCl. The variation of the pH of 5 per cent solutions of these two amino-acids between the limits of 5.0 and 1.16 had no measurable influence on the viscosity of the solution. This cast a serious doubt on the assumption that the variations in the curve of the viscosity of gelatin, as expressed in Fig. 1, were caused by variations in the hydration of the individual gelatin ions.[13]

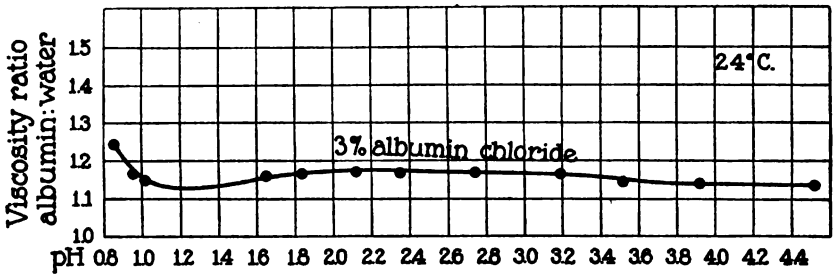

FIG. 2. Showing that solutions of crystalline egg albumin have a low viscosity in comparison with gelatin solutions, and that the pH has little influence on the viscosity of solutions of crystalline egg albumin at pH over 1.0 and at ordinary temperature.

This doubt was increased by experiments on the influence of pH on the viscosity of crystalline egg albumin which gave also a practically negative result. Fig. 2 gives such an experiment with 3 per cent originally isoelectric albumin brought to different pH through the addition of HCl. The ordinates are the viscosity ratios of albumin solution over water, drawn on a larger scale than those in Fig. 1, and the abscissæ are the pH of the solution. It is obvious that the pH has only a very slight if any influence on the viscosity of solutions of crystalline egg albumin between pH 4.6 and pH 1.0. With a further lowering of pH the viscosity suddenly rises, a fact to which we shall return later. It is also obvious that the viscosity

[13] These experiments were carried out by Dr. Elizabeth Brakeley.

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ratio, protein solution: water, is considerably smaller in the case of albumin solutions than in the case of gelatin solutions.

The method of the experiments was as follows. 50 cc. of a 6 per cent solution of isoelectric crystalline egg albumin were mixed with 50 cc. of HCl solution of different concentration and the pH measured. The solution was rapidly brought to a temperature of 24°C. and the viscosity was measured immediately at that temperature.

The question then arises, why do amino-acids and at least one protein, namely crystalline egg albumin, behave so differently from gelatin in regard to the influence of the pH on the viscosity? As long as we assume that the influence of the hydrogen ion concentration on the viscosity of gelatin-acid salt solution is due to the hydration of the individual protein ions this difference is incomprehensible since the amino-acids as well as crystalline egg albumin should in this case show the same influence of ionization on hydration as the gelatin.

The puzzle becomes still greater if we take into consideration the fact that the osmotic pressure of solutions of crystalline egg albumin is affected in the same way by the hydrogen ion concentration as is the osmotic pressure of gelatin solutions. Why then do these two proteins behave so differently as regards the influence of the pH on their viscosity?

To answer this question we are forced to the conclusion that gelatin in solution must possess a way of increasing its volume which is lacking in the case of solutions of crystalline egg albumin (at least at ordinary temperature and at a pH above 1.0). This difference seems to be connected with a difference in the ability to form a gel. Solutions of isoelectric crystalline egg albumin of a high concentration can be kept for many months at a temperature just above the freezing point without setting to a jelly or without even showing an increase in viscosity; while solutions of isoelectric gelatin of even a low concentration show a rapid increase in viscosity and may set to a jelly under the same conditions of temperature and pH which do not alter the viscosity of egg albumin. Moreover, crystalline egg albumin has a very low viscosity compared with that of the same mass of gelatin in solution.

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When, however, the pH of a 3 per cent solution of crystalline egg albumin falls to 0.85 or below, the solution can set to a gel and in that case its viscosity rises to the same order of magnitude as the viscosity of gelatin solutions except that an increase in temperature has the opposite effect as in the case of gelatin solutions. This is illustrated by Figs. 3 and 4. Both figures show the influence of time on the

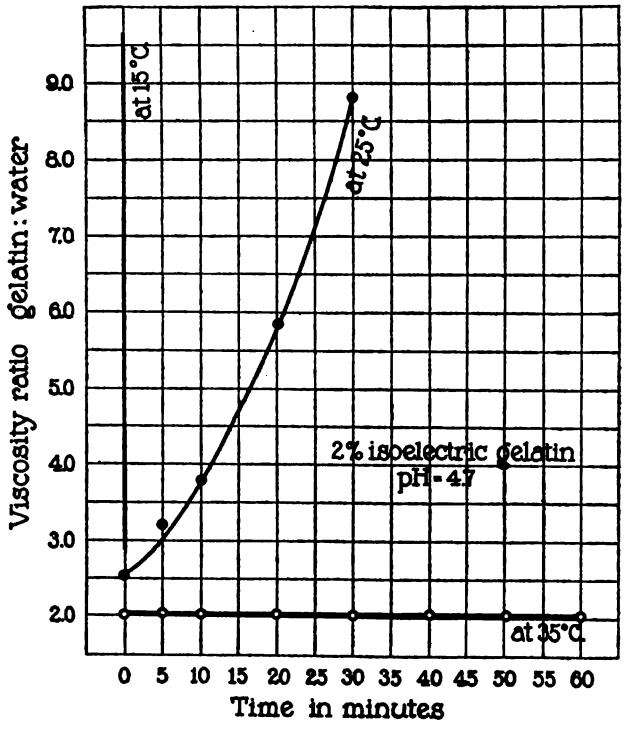

FIG. 3. Influence of time on viscosity of isoelectric gelatin solutions at different temperatures.

viscosity ratio of solutions of gelatin or albumin to that of pure water. Fig. 3 shows that the viscosity of a 2 per cent solution of isoelectric gelatin rises rapidly at 15°C., more slowly at 25°C., and quite slowly at 35°C.

Fig. 4 gives the influence of time on the viscosity of 3 per cent solutions of albumin chloride at pH 0.85 where the solutions are

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opalescent and have a tendency to set to a gel. The reader will notice that at that pH viscosity of solutions of albumin chloride behaves like the viscosity of gelatin solutions, inasmuch as the viscosity of albumin chloride solutions also rises with time as soon as

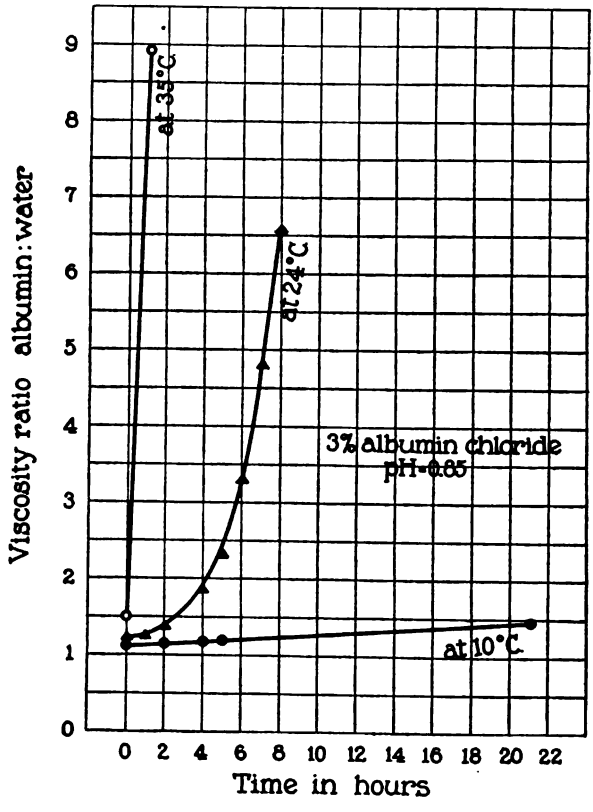

FIG. 4. Influence of time on viscosity of solutions of crystalline egg albumin of pH 0.85 at different temperatures. At this pH the albumin chloride solutions have a tendency to set to a jelly and have the same high order of viscosity as the gelatin solutions.

the solution acquires the property of setting to a gel, and that the order of magnitude of the viscosity of albumin chloride solutions capable of setting to a gel no longer differs from that of gelatin solution. The only difference is that of the influence of temperature on the viscosity which is the reverse in the case of the two proteins.

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High temperature favors jelly formation in the case of egg albumin and retards it in the case of gelatin.

These observations corroborate the suspicion that the high order of magnitude of viscosity of gelatin solutions may be in some way connected with the tendency of this protein to set to a gel.

We do not yet know how the tendency of a protein solution to form a gel can account for the following two facts: first, that this tendency is accompanied by a rise in viscosity, and second, that the pH influences the viscosity in a way suggestive of the Donnan equilibrium. One possible answer to this question might be that the formation of a continuous gel by a protein solution may be preceded by the formation of a number of submicroscopic particles of gel, each occluding a considerable amount of water. This occlusion of water would cause a considerable increase in the volume of the mass of gelatin and this could account for the rise in viscosity with the tendency to form a gel. The Donnan equilibrium would regulate the quantity of water occluded by each particle and this would account for the influence of pH. The idea of such a possibility gave rise to the following experiments on the viscosity of suspensions of powdered gelatin in water.

III. Influence of Volume of Powdered Gelatin on Viscosity.

When we suspend finely powdered gelatin in water of a sufficiently low temperature, and measure the viscosity of such suspensions we find that they may have even a higher viscosity than gelatin solutions of the same concentration of gelatin and that the pH influences the viscosity of the suspension in the same characteristic way as that expressed in the curves of Fig. 1.

0.5 gm. of Cooper's powdered commercial gelatin of a pH of about 6.0 was added to 100 cc. of water containing varying amounts of HCl. The particles had uniform size (going through Sieve 100 but not through Sieve 120), but their shape was extremely irregular. They were left in the solution several hours at 20°C., and then their time of outflow through a capillary tube was ascertained at 20°C. The time of outflow of water through the viscometer at this temperature was 24 seconds. It was essential to stir the suspension thoroughly

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before sucking it into the viscometer since the gelatin particles sink rapidly to the bottom of the dish.

After the viscosity measurements were taken, the suspension was put on a filter of cotton wool and the supernatant water allowed to drain off. By measuring the volume of the filtrate and deducting this from the original volume of the suspension (which was in all cases 100 cc.), the volume of the gelatin (with some error) was obtained. Then the gelatin was melted, and the pH of the melted mass of gelatin as well as of the filtrate was determined potentiometrically. Fig. 5 gives the result of such an experiment. The lower

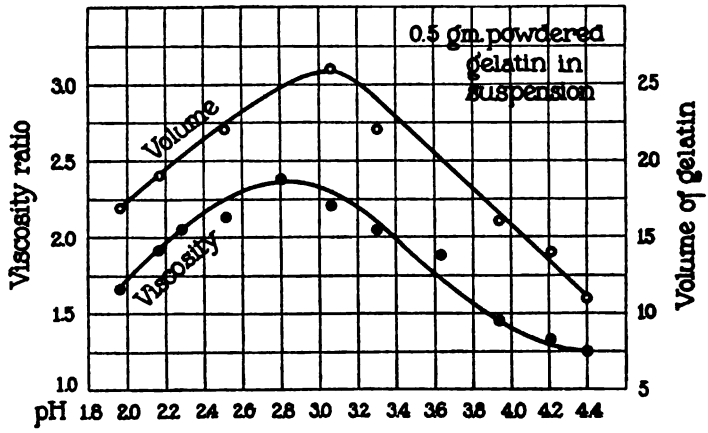

FIG. 5. Showing that the influence of pH on viscosity of 0.5 per cent suspensions of powdered gelatin in water is similar to the influence of pH on viscosity of gelatin solutions, and that the volume occupied by the particles in the suspension varies in a similar way as the viscosity. Temperature 20°C.

curve shows the influence of the pH (of the gelatin) on the viscosity, and the upper curve the influence of the pH on the volume of the gelatin. The two curves are similar.

Moreover, the viscosity values for the suspensions of 0.5 gm. of powdered particles in water are greater than the viscosity values of a 0.5 per cent solution of freshly liquefied gelatin for equal pH.[14] The highest viscosity ratio of the 0.5 per cent gelatin solution was about 1.75 while the highest viscosity ratio for the 0.5 per cent suspension of gelatin was about 2.4 (Fig. 5).

[14] The viscosity of the solution increases on standing.

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These experiments prove, first, that a suspension of powdered gelatin in water shows the same variation in viscosity with the variation of the hydrogen ion concentration as does a solution of freshly prepared gelatin; and, second, that the relative volume of the suspended particles varies in a similar way as the viscosity (Fig. 5). In this case there is little doubt that the variations in the volume of the suspended particles of gelatin under the influence of the pH are due to the existence of a Donnan equilibrium between the particles and the surrounding water, since we have already shown in a former publication that there exists a difference in the pH of the solid particles of powdered gelatin and the supernatant water and this fact was further corroborated in these experiments (Table I).

TABLE I.

Donnan Equilibrium Between Suspended Particles of Gelatin and Supernatant Water After 20 Hours.

0.5 gm. of Gelatin Suspension in 100 cc. H_2O Containing Various Amounts of HCl at Temperature of 20°C.

pH of gelatin particles	4.79	4.62	4.33	4.17	3.93	3.60	3.26	3.02	2.68	2.39	2.16	2.07	1.80
pH of supernatant water	4.74	4.30	3.95	3.76	3.55	3.21	2.95	2.77	2.56	2.31	2.10	2.02	1.75

The point which is of importance is the question of the applicability of Einstein's formula to these experiments

$$\frac{\eta}{\eta_0} = 1 + 2.5\varphi$$

The fact that the shape of the suspended particles of gelatin is very irregular and that the average size of the individual particles plays also a rôle must warn us not to expect too strict an applicability of the formula in our case; and we may expect to obtain slightly different values than 2.5 for the constant. Since we can measure $\frac{\eta}{\eta_0}$ as well as φ directly in our experiments, we may write Einstein's equation in the form

$$\frac{\eta}{\eta_0} - 1 = c\varphi$$

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and may try to calculate the value of $c = \frac{\frac{\eta}{\eta_0} - 1}{\varphi}$ from our observations. Since φ is the ratio of the volume of the gelatin to the volume of the solution and the latter is 100 cc., we have to multiply the value $\frac{\frac{\eta}{\eta_0} - 1}{\text{volume of gelatin}}$ by 100 to obtain c.

Table II shows the results of such a calculation.

TABLE II.

pH of gelatin.	$\frac{\eta}{\eta_0}$	Volume of gelatin.	c
		cc.	
4.80	1.070	4.5	1.5
4.40	1.250	8.0	3.1
4.21	1.345	11.0	3.1
3.94	1.515	16.0	3.2
3.63	1.845	18.0	4.2
3.30	2.120	21.0	5.3
2.80	2.340	22.5	6.0
2.51	2.150	19.5	5.9
2.28	2.080	18.0	6.0
2.16	1.865	16.0	5.4
1.96	1.726	16.0	4.5

Considering the fact that the gelatin particles are not perfect spheres, as Einstein's theory demands, and considering the further fact that the measurements of the volume of gelatin are crude, it is surprising that where the volume of the gelatin is small the constant c is so near that expected on the basis of Einstein's formula, namely 3.1 instead of 2.5. Larger values are found (from 4.2 to 6.0) when the swelling of the gelatin particles becomes too large to permit the strict application of Einstein's formula. In all probability a second variable enters in this case, namely the large size of the individual granules. We shall see in a later paper that in the case of suspensions of gelatin particles the viscosity is not only a function of the relative volume of the suspended particles but also of their size, increasing (for the size used in our experiments) with the size. This might account for the fact that in Table II the value of c varies with the size of the particles.

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If we consider all these complicating circumstances there can be little doubt left that the influence of pH on the viscosity of suspensions of particles of gelatin is mainly due to the change in volume of these particles under the influence of the pH and that this change of volume finds its explanation in the Donnan equilibrium between the particles and the surrounding liquid.

It is well known, and it has been discussed in preceding papers,[15] that the viscosity of a gelatin chloride solution, *e.g.*, of pH 3.0, is

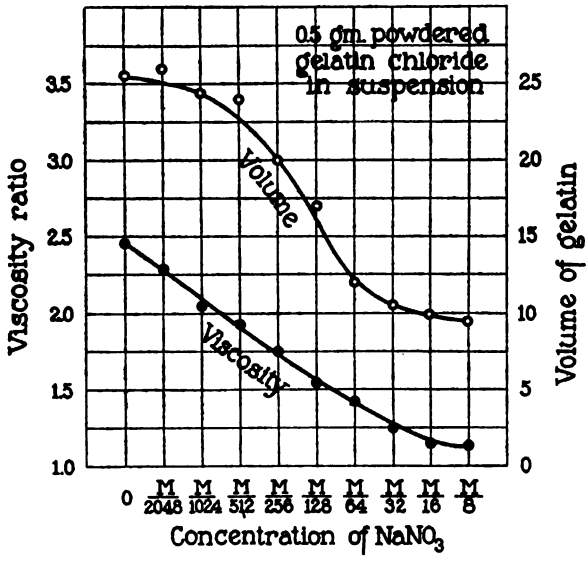

FIG. 6. Showing depressing influence of neutral salts on viscosity of suspensions of powdered gelatin in water and on the volume occupied by the gelatin particles in the suspension.

lowered when neutral salts are added and the pH kept constant. The same is true for the viscosity of suspensions of powdered gelatin. Doses of 0.5 gm. of powdered gelatin of pH 6.0, going through Sieve 100 but not through Sieve 120, were put each into 100 cc. of water containing 6 cc. of 0.1 N HCl, and different quantities of $NaNO_3$, so that the concentration of the salt varied in the different solutions from M/8 to M/2048. One solution contained no salt. The pH of the

[15] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 391.

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gelatin varied in the neighborhood of 3.0; the temperature was 20°C. After 2½ hours, when the Donnan equilibrium between the particles and the surrounding solution was supposed to be established, the viscosity of each suspension was measured at 20°C. and the volume occupied by the suspended particles of gelatin was ascertained in the manner described. It was found that the addition of salt diminished the relative volume of the gelatin particles and the viscosity in a similar way (Fig. 6). The observed volume of the solutions containing little or no salt was probably a little too great on account of incomplete filtration.

The measurement of the pH of the gelatin solution and the outside solution showed that the addition of salt diminished the difference between the two, as Donnan's theory demands (Table III).

TABLE III.

	Concentration of $NaNO_3$.								
	0	m/2,048	m/1,024	m/512	m/256	m/128	m/64	m/32	m/16
pH of gelatin particles	3.04	3.04	3.03	3.02	3.00	3.02	2.97	2.94	2.85
pH of supernatant liquid	2.74	2.76	2.76	2.76	2.77	2.80	2.78	2.77	2.70
Difference, pH inside minus pH outside	0.30	0.28	0.27	0.26	0.23	0.22	0.19	0.17	0.15

SUMMARY AND CONCLUSION.

1. Gelatin solutions have a high viscosity which in the case of freshly prepared solutions varies under the influence of the hydrogen ion concentration in a similar way as the swelling, the osmotic pressure, and the electromotive forces. Solutions of crystalline egg albumin have under the same conditions a comparatively low viscosity which is practically independent of the pH (above 1.0). This difference in the viscosities of solutions of the two proteins seems to be connected with the fact that solutions of gelatin have a tendency to set to a jelly while solutions of crystalline egg albumin show no such tendency at low temperature and pH above 1.0.

2. The formulæ for viscosity demand that the difference in the order of magnitude of the viscosity of the two proteins should cor-

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respond to a difference in the relative volume occupied by equal masses of the two proteins in the same volume of solution. It is generally assumed that these variations of volume of dissolved proteins are due to the hydration of the isolated protein ions, but if this view were correct the influence of pH on viscosity should be the same in the case of solutions of gelatin, of amino-acids, and of crystalline egg albumin, which, however, is not true.

3. Suspensions of powdered gelatin in water were prepared and it was found, first, that the viscosity of these suspensions is a little higher than that of gelatin solutions of the same concentration, second, that the pH influences the viscosity of these suspensions similarly as the viscosity of freshly prepared gelatin solutions, and third, that the volume occupied by the gelatin in the suspension varies similarly as the viscosity which agrees with the theories of viscosity. It is shown that this influence of the pH on the volume occupied by the gelatin granules in suspension is due to the existence of a Donnan equilibrium between the granules and the surrounding solution.

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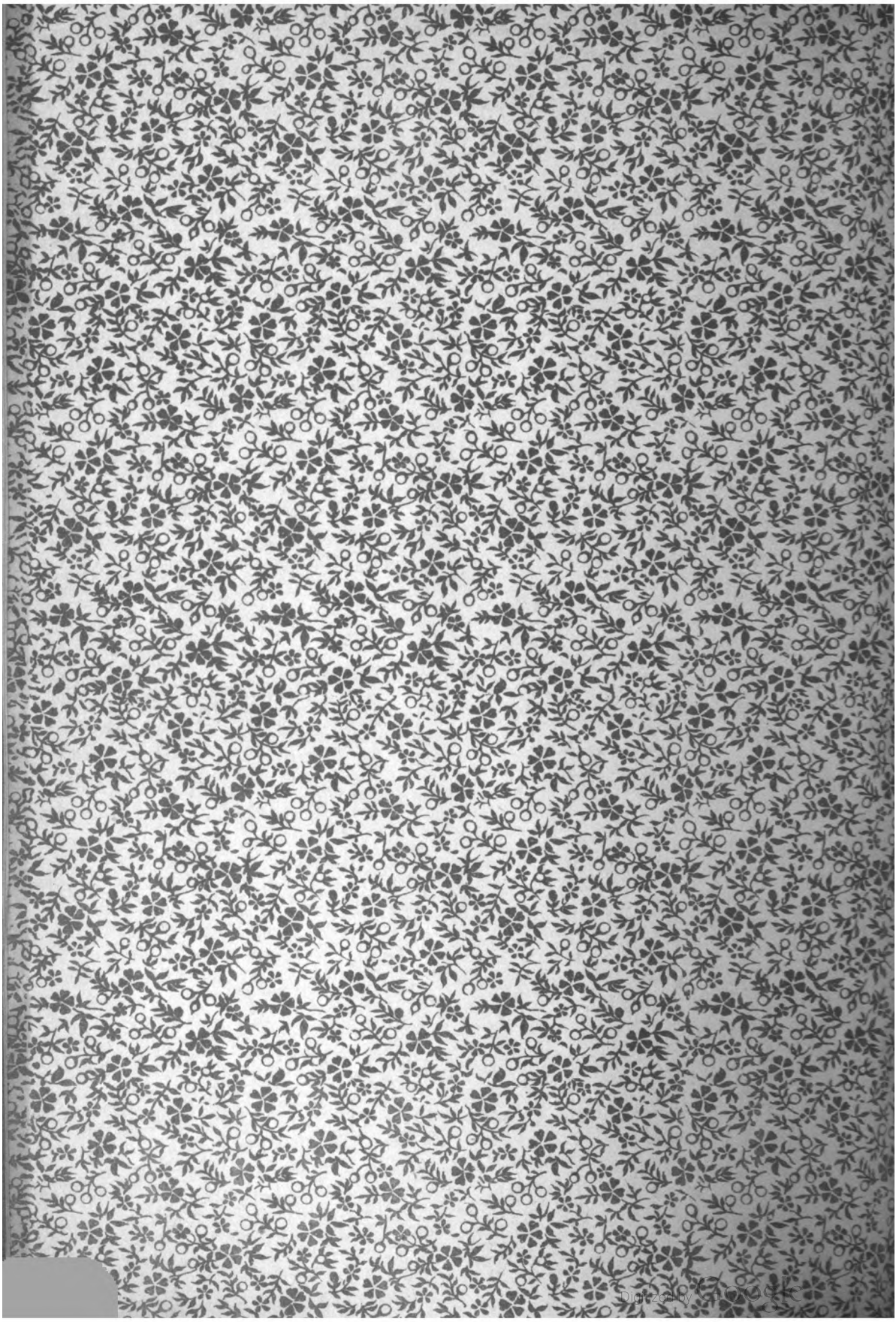

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